

## Abstract

### Fluoride Toxicity and the Cellular Response in Normal, Sensitized, and Resistant Yeast

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Fluoride is abundant in the air, soil, and water. High levels of fluoride activate cell cycle arrest and apoptosis; however, the mechanism of fluoride's toxicity remains unknown. Fluoride has a high affinity for metals, and *in vitro* fluoride has been demonstrated to inhibit over one hundred metallo-proteins at millimolar fluoride concentrations. Bacteria and other microbes remove intracellular fluoride through protein channels. Deletion of fluoride exporter genes (FEX) in *S. cerevisiae* results in cell cycle arrest at 1000-fold lower extracellular fluoride concentrations, well below the  $K_D$  of inhibition for any tested metallo-proteins. Consequently, this strain serves as a powerful tool to study intracellular fluoride toxicity. I conducted several genetic screens to elucidate both the mechanism by which fluoride activates cell cycle arrest, as well as how cells can defend themselves against fluoride. Through an overexpression screen and RNA-Seq analysis, I found that fluoride disrupt the plasma membrane pH gradient, resulting in the inhibition of nutrient uptake and the activation of starvation signaling. Supplementation with nutrients, particularly phosphate, partially rescues from fluoride toxicity. I identified this as a nonspecific effect, and observed that phosphate only rescues fluoride toxicity in organisms lacking FEX. I also selected and sequenced 81 strains with 100-1200-fold increased resistance to fluoride. These strains were heavily mutated in nutrient uptake and flocculation genes. I found that overall, these strains had an acidified cytoplasm that reversed the accumulation of intracellular fluoride.

In wild type yeast, genes involved in the resistance to fluoride toxicity were found to be widely conserved in resistance against other acid toxicity, with the exception of Golgi-mediated vesicle transport. The Golgi has been widely reported to be inhibited by metallo-fluoride; this is consistent with fluoride toxicity being distinct from other acids in wild type yeast due to its affinity with metal. I also observed that fluoride activates cell cycle arrest at lower intracellular concentrations in yeast lacking FEX. After further investigation, I found that FEX has an additional role in acid resistance. It provides resistance to outside acidity, independent of fluoride. This effect was found to be dependent on pore one of FEX, previously thought to be nonfunctional.

In all, I have identified a bimodal pattern of fluoride toxicity: in yeast lacking FEX, the predominant mechanism of fluoride toxicity was linked to its effect as a weak acid, disrupting the electrochemical potential and activating nutrient starvation signaling. FEX was found to play a role in resisting acid toxicity, and yeast expressing FEX showed toxicity responses more consistent with metallo-protein inhibition. This role of FEX in acid resistance is conserved in higher eukaryotes including plants and sea sponges. While FEX has not yet been identified in humans, investigating whether fluoride toxicity is driven primarily by acid toxicity or metallo-protein inhibition could elucidate the existence of an analogous protein.

Fluoride Toxicity and the Cellular Response in Normal, Sensitized, and Resistant Yeast

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by  
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## Chapter 1: Overview of fluoride toxicity

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### **Abstract:**

Fluoride is ubiquitously present throughout the world. It is released from minerals, magmatic gas, and industrial processing, and travels in the atmosphere and water. Exposure to low concentrations of fluoride increases overall oral health. Consequently, many countries add fluoride to their public water supply at 0.7-1.5 ppm. Exposure to high concentrations of fluoride, such as in a laboratory setting often exceeding 100 ppm, results in a wide array of toxicity phenotypes. This includes oxidative stress, organelle damage, and apoptosis in single cells, and skeletal and soft tissue damage in multicellular organisms. The mechanism of fluoride toxicity can be broadly attributed to four mechanisms: inhibition of proteins, organelle disruption, altered pH, and electrolyte imbalance. Recently, there has been renewed concern in the public sector as to whether fluoride is safe at the current exposure levels. In this review, we will focus on the impact of fluoride at the chemical, cellular, and multisystem level, as well as how organisms defend against fluoride. We also address public concerns about fluoride toxicity, including whether fluoride has a significant effect on neurodegeneration, diabetes, and the endocrine system.

### **I. Chemical properties of fluoride**

The element fluorine has the highest electronegativity and the second highest electron affinity, making it highly reactive. At room temperature, fluorine exists as the gas F<sub>2</sub>, which reacts explosively with many elements. Fluorine is so reactive that it can form complexes with noble gases, most notably xenon (Holloway 1966). Due to its reactivity and low stability, isolated fluorine is never found in nature. Instead, fluorine is either found as a complex or in its ionized form, fluoride.

Fluoride interacts with many cations, including hydrogen and a wide variety of metals. It is the only halide with a positive pK<sub>a</sub> (3.2), and therefore exists in acidic environments as its protonated form (HF). HF, commonly released as industrial or volcanic fumes, turns gaseous above 20°C. Fluoride is most toxic in its

protonated form, and vertebrates that reside in areas near HF production often show symptoms of lung damage and fluoride toxicity.

Fluoride readily associates with metals. This affinity is driven by three factors: negative Gibbs free energy for formation, a high stability constant, and poor solubility of the metal-fluoride complex (Table 1).

	Stability Constant ( $K_f = [ML]/[M][L]$ )		Gibbs Free Energy (KJ/mol)		Solubility ( $K_{sp} = g/100g H_2O$ )	
	Cl <sup>-</sup> ( $K_f$ )	F <sup>-</sup> ( $K_f$ )	Cl <sup>-</sup> ( $\Delta_f G^\circ(c)$ )	F <sup>-</sup> ( $\Delta_f G^\circ(c)$ )	Cl <sup>-</sup> ( $K_{sp}$ )	F <sup>-</sup> ( $K_{sp}$ )
Al(F,Cl) <sub>3</sub>	9×10 <sup>-2</sup>	5×10 <sup>6</sup>	-628.8	-1431.1	45.1	0.5
Fe(F,Cl) <sub>3</sub>	7×10 <sup>-1</sup>	2×10 <sup>5</sup>	-334.0	-668.6	91.2	5.92
Be(F,Cl) <sub>2</sub>	5×10 <sup>-1</sup>	1×10 <sup>3</sup>	-445.6	-979.4	71.5	-
Mn(F,Cl) <sub>2</sub>	4×10 <sup>-1</sup>	8×10 <sup>2</sup>	-440.5	-	77.3	1.02
Mg(F,Cl) <sub>2</sub>	7×10 <sup>-1</sup>	5×10 <sup>2</sup>	-591.8	-1071.1	56.0	0.013
Zn(F,Cl) <sub>2</sub>	9×10 <sup>-1</sup>	2×10 <sup>1</sup>	-369.4	-713.3	408	1.55
Ca(F,Cl) <sub>2</sub>	7×10 <sup>-1</sup>	1×10 <sup>1</sup>	-748.8	-1175.6	81.3	0.0016
Ni(F,Cl) <sub>2</sub>	9×10 <sup>-1</sup>	5×10 <sup>0</sup>	-259.0	-604.1	67.5	2.56

**Table 1: Energetics of fluoride and chloride bound to metals** (Agarwal *et al.* 1971; Cadek *et al.* 1971; Bond *et al.* 1980; Hefter *et al.* 1984; Fovet *et al.* 2000; Chadhuri *et al.* 2001; Morris *et al.* 1965; Gross *et al.* 1973; Gaizer *et al.* 1980; Maher *et al.* 1982; Palmer *et al.* 1993; Harris *et al.* 2011).

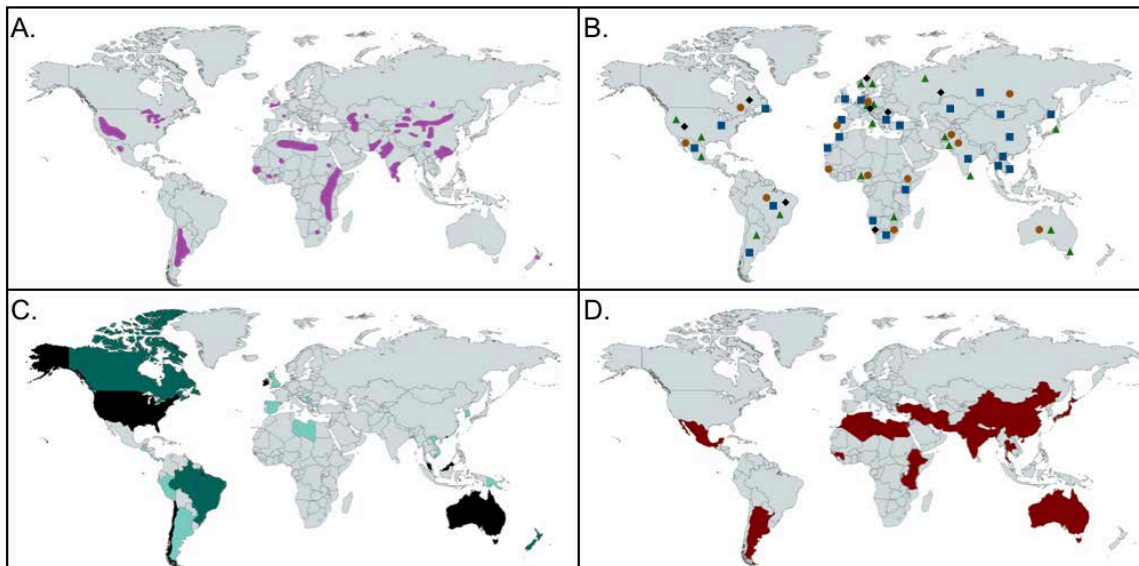
The energy state and stability of metallo-fluoride is much greater than that of other metallo-halides, and as such fluoride can often displace metal interacting partners in nature. The most favorable metal reactions with fluoride are aluminum, calcium, and magnesium, while the most stable are aluminum, iron, and beryllium. Overall, the most favorable metal to bind fluoride is aluminum (Skelton 1971; Martin 1988; Martin 1996). Aluminum is also the most abundant metal on the earth's crust, and only micromolar levels of aluminum are necessary to complex with fluoride (Mullenix 2014; Berger *et al.* 2015). In terms of biological

relevancy, calcium and magnesium are at much higher abundance and form complexes with fluoride *in vivo* (Marier 1980; Spencer *et al.* 1980). Interestingly, both of these metals are highly insoluble when complexed with fluoride. As calcium- or magnesium-fluoride precipitates out of solution, these complexes do not readily associate with other elements.

## **II. Fluoride's occurrence in nature**

Fluoride gradually accumulates in the environment from volcanic emissions, dissolution of minerals, and industrial byproducts (WHO 2002). Fluoride is present at roughly 300-900 ppm throughout the earth's crust, and is estimated to be 200-fold more abundant in the mantle (Weinstein and Davison 2004; Koga and Rose-Koga 2018). Therefore, magma and magmatic gas contain fluoride, both unbound and complexed with hydrogen, silicon, and ammonium (Symonds *et al.* 1988; Das and Behera 2008). These molecules settle as ash into the nearby soil and groundwater. Monitoring of fluoride particles in ash from the 1991 eruption of Mt. Hudson in Chile found that fluoride was highest in ash deposited furthest from the volcano; fluoride completely dissolved out of the ash and seeped into the surrounding soil and water sources after the first rainfall (Rubin *et al.* 1994). Historically, organisms in areas with high volcanic activity have displayed signs of fluoride poisoning (Olsen and Fruchter 1986; Witham *et al.* 2005; Fluek and Smith-Fluek 2013). Today, volcanic regions with high fluoride include Sicily, New Zealand, Iceland, the East African Rift, China, and South India (Cronin *et al.* 2003; Bellomo *et al.* 2006; D'Alessandro 2006).

Fluorine can also be found in 296 different species of minerals, the most abundant of which are fluor spar ( $\text{CaF}_2$ ), fluorapatite ( $\text{Ca}_5(\text{PO}_4)_3\text{F}$ ), topaz ( $\text{Al}_2(\text{SiO}_4)(\text{F},\text{OH})_2$ ), and cryolite ( $\text{Na}_3\text{AlF}_6$ ) (Mineralogy Database; Garcia and Borgnino 2015). These occur as vein deposits associated with igneous rocks, especially metallic minerals. Some of the largest deposits of fluoro-minerals are found in China, Mexico, Mongolia, South Africa, and Russia; this occurrence correlates strongly with areas whose populations suffer from endemic fluoride toxicity (Fig. 1) (Kilgore and Pelham 1987; Gupta and Ayoob 2016). Endemic fluoride toxicity in humans occurs from the chronic consumption of over 1.5 ppm (75  $\mu\text{M}$ ) fluoride.



**Figure 1: Global distribution of fluoride.** Shown here are areas with (A)  $< 1.5$  ppm fluoride in the groundwater (purple), (B) mining of fluoro-compounds (blue squares - fluor spar, brown circles - fluorapatite, green triangles - topaz, and black diamonds - cryolite), (C) government-regulated fluoridated water (light teal – 0-33%, teal – 34-67%, black – 68-100% of population), and (D) endemic fluoride toxicity (red) (Unicef 1999, WHO 2004, Gupta and Ayoob 2016, British Geological Survey, and the USGS Database).

Demineralization leads to an increased concentration of fluoride in the soil and water. Over time, minerals naturally break down through weathering and erosion. Most fluoride-metal complexes, especially fluor spar and fluorapatite, are poorly soluble in water (Moreno *et al.* 1974; Pan and Darvell 2007). Without further dissolution, the fluoride-metal complexes distribute into the soil where they are absorbed by plants and microbes. However, alkaline groundwater ( $\text{pH} > 8$ ) can solubilize fluoride from minerals. The East African Rift Valley has several alkaline lakes near volcanic sites; there fluoride concentrations in groundwater range from 2.1-9.0 ppm (100-500  $\mu\text{M}$ ) (Malago *et al.* 2017). Fluoride dissolution from minerals is also favored under conditions with geothermal water, low concentrations of calcium and other metals, and areas with strong evaporation (Jha *et al.* 2013; Bouzourra *et al.* 2015; Batabyal and Gupta 2017). Acid rain has similar effects. The acidity releases sodium bicarbonates into the water, which in turn react with and dissolve fluoride minerals (Apambire *et al.* 1997; Nath and Dutta 2010; Salifu *et al.* 2012). Over time, fluoride eventually deposits into the ocean. Oceanic water naturally contains fluoride, which cycles in and out of the



atmosphere through the water cycle (Carpenter 1969). This fluoride is thought to be from the breakdown of marine sediments, such as phosphate rock. Free fluoride is present in the ocean at 1.2-1.4 ppm (60-80  $\mu\text{M}$ ) (WHO 2004).

Fluoride is also released into the air, water, and soil during mining and industrial processes. Fluoride is either used in a reaction, such as in aluminum smelting or glass production, or released as a byproduct, such as in phosphate fertilizer production, ceramic production and coal burning (Monfort *et al.* 2008; Gouider *et al.* 2010; Seixas *et al.* 2010). From these processes, excess fluoride is released into the environment as fumes or in groundwater (Jaccoud *et al.* 2000; Larsson *et al.* 2017; Roy *et al.* 2017). Phosphate fertilizer is a particular problem for widespread fluoride groundwater contamination, as fluoride is both released during the breakdown of phosphate ore, and makes up an additional 1.5-3% of the final fertilizer, which then enters the environment (McLaughlin *et al.* 1996; Li *et al.* 2015). For fluoride released as fumes, coal burning poses a substantial health risk. Fluoride is emitted as fumes upon coal combustion. In areas with indoor coal usage, many individuals show signs of fluoride toxicity (Li and Cao 1994; Ando *et al.* 1998; 2001). In 1997, an estimated 31 million people in China showed signs of fluoride toxicity from coal intake (Hou 1997, Ando *et al.* 2001). Fluoride emitted as gas enters the atmosphere, where it has an approximate concentration of 0.08-1.61  $\mu\text{g}/\text{m}^3$  in areas like Beijing (Feng *et al.* 2003). Fluoride travels efficiently through the air before precipitating back into the soil and water, so fluoride emissions can lead to higher fluoride exposure in areas far from the original industrial site (Walna *et al.* 2013; Walna 2015).

Fluoride exposure can also vary based on diet. All foods contain fluoride. Some of the highest concentrated food sources occur from fluoride-accumulating plants, such as tomato, spinach, grapes, tea, and elderberry. The Hitchcock lab found that tomatoes grown in 10 mM NaF (190 ppm) accumulated 900 ppm fluoride (dry weight) in their leaves (Jacobsen *et al.* 1966). However, the amount of fluoride in plants depends strongly on the fluoride concentration in the soil; U.S.-grown tomatoes typically contain around 0.02 ppm fluoride. Plants such as tea accumulate fluoride with age. In populations with heavy tea consumption, fluoridated tea is believed to be the primary mechanism of adult fluoride toxicity (Cao *et al.* 2003). In many developed countries, a large majority of fluoride exposure comes from government-instituted fluoridated water (0.7-1.5 ppm), toothpaste (typically 1,000-2,500 ppm), dental gel (12,300 ppm) and varnish (23,000 ppm) (Cappelli and Mobley 2008; Pretty 2016).

### III. Benefits of water fluoridation

The practice of adding fluoride to the public water supply has, in general, increased overall dental health. The first documentation of fluoride's effects began with three independent reports from Italy, America, and the U.K. in the early 1900s noting that individuals with mottled, brown-stained teeth had lower incidences of dental caries (Eager 1901; McKay 1917; Ainsworth 1933). H.V. Churchill, who originally attributed the effect to high aluminum exposure, later found fluoride to be the causative agent preventing caries and triggering the skeletal defect later known as "fluorosis" (Churchill 1931). Fluoride was first added to the public water supply in Grand Rapids, Michigan in 1945 as a method of caries prevention. As of 2019, 24 countries participate in water fluoridation. An estimated 30-60% of caries incidences have been reduced because of water fluoridation (Armfield 2010). Because of this advancement, the CDC named water fluoridation one of the top ten greatest public health achievements of the twentieth century (CDC 1999).

Controlled exposure to fluoride increases overall teeth quality through enamel replacement and the killing of plaque-causing bacteria. Fluoride reacts with tooth enamel because of its high affinity to metals. Tooth enamel is comprised mostly of the mineral hydroxyapatite ( $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ). Fluoride readily displaces the hydroxide to form fluorapatite ( $\text{Ca}_5(\text{PO}_4)_3\text{F}$ ) (Fig. 2). Under normal conditions bacteria like *Streptococcus mutans* ferment along the enamel, producing acid that gradually dissociates the hydroxyapatite (critical pH 5.5) (Featherstone 2008). When fluoride is present, it scavenges excess phosphate and calcium in the saliva for partial tooth remineralization, as well as displaces the hydroxyl group in remaining enamel (Featherstone 1999; Amaechi and van Loveren 2013). The resulting fluorapatite is more resistant to acidity (critical pH 4.5) than normal enamel, and as such, individuals exposed to fluoride have less tooth decay and more enamel than individuals with no fluoride exposure (Slade *et al.* 2018). For this reason, fluoride is added to the water sources of many countries.

### IV. Toxicity from the interaction of fluoride with metals

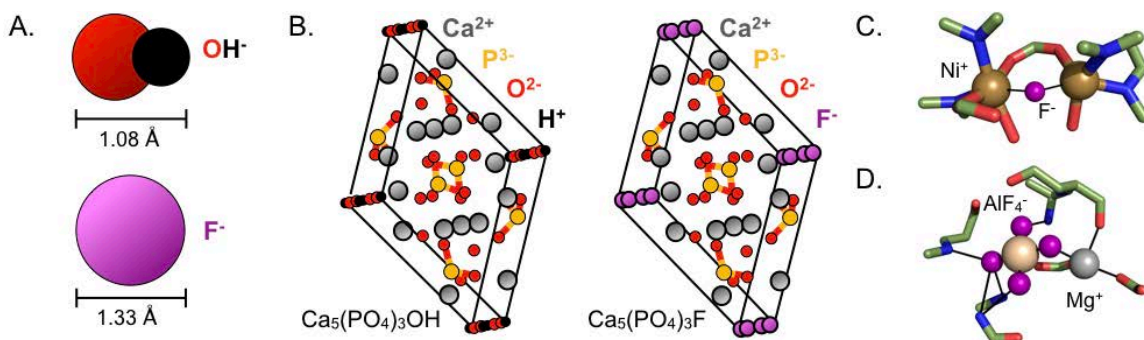
While low doses of fluoride are beneficial for overall teeth integrity, high doses of fluoride lead to a myriad of stress phenotypes. Broadly, fluoride triggers oxidative stress, cell cycle arrest, and apoptosis. While the exact mechanism of fluoride toxicity is unknown, the stress phenotypes are generally attributed to the inhibition of proteins, organelle disruption, altered pH, and electrolyte imbalance (Adamek *et al.* 2005; Barbier *et al.* 2010; Agalakova *et al.* 2011). These four mechanisms occur to varying degrees depending on

the concentration of fluoride, its route of administration in multicellular organisms, and a cell's surrounding environment. There is not a complete consensus over which downstream stress phenotype is linked to each mechanism, as each stressor can independently trigger oxidative stress and apoptosis. However, most papers attribute the primary mechanism of fluoride toxicity with its ability to inhibit metalloproteins (Adamek *et al.* 2005; Agalakova *et al.* 2011). A common practice in the study of fluoride toxicity is to look for reduced activity in an organelle or pathway under fluoride exposure, and then find essential metalloproteins within that organelle/pathway. However, many pathways show altered rates at lower fluoride exposure than that needed to inhibit essential proteins *in vitro*. This suggests either a target that is still unestablished, or that fluoride initiates toxicity through a combination of many targets.

#### Inhibition of metalloproteins by ionized fluoride

Fluoride's properties as a protein inhibitor were established prior to the discovery of its properties in preventing dental caries. Starting in the late 1800s, sodium fluoride was identified as a lipase inhibitor (Tappeiner 1889; 1890, Kastle and Loevenhart 1900; Loevenhart and Pierce 1906). Later fluoride was found to inhibit a range of phosphatases, kinases, hydrolases, and other metalloproteins *in vitro*. As of 2018, the protein databank has collected over 700 proteins crystallized in complex with fluoride (RCSB). Over 100 of these have had separate, independent enzymatic studies for fluoride inhibition *in vitro* (Adamek *et al.* 2005). While fluoride has been reported in a few select cases to bind directly to amino acids or to displace essential hydroxides, the majority of noted protein interaction is through either (1) fluoride binding to an essential metal in a metalloprotein's active site, or (2) the complexing of fluoride with metal and acting as a substrate mimic (Edwards *et al.* 1984; Adamek *et al.* 2005; Schenk *et al.* 2008).

An estimated 30-50% of proteins require metal; consequently, there are thousands of potential targets for fluoride inhibition (Ascone *et al.* 2003). Fluoride is negatively charged, and associates with positive sites on proteins (Fig. 2).



**Figure 2: Fluoride interactions *in vitro*.** (A) Fluoride is the most similar in terms of size and charge to hydroxide, but has a much higher affinity for metals. (B) unit cells of crystallized hydroxyapatite ( $\text{Ca}_5(\text{PO}_4)_3\text{OH}$ ) and fluorapatite ( $\text{Ca}_5(\text{PO}_4)_3\text{F}$ ) [Minerology Database]. (C) Crystal structures of fluoride bound to urease and (D) phosphoserine phosphatase. Structures were generated on PyMOL using RCSB PDB (C) 4GOA and (D) 1L7N.

In the case of metalloproteins, fluoride interacts with the essential metals, forming a highly stable, often insoluble complex. Fluoride can also form ternary complex with metal and phosphate, which has even greater stability than metallo-fluoride (Qin *et al.* 2006). Many pathways, particularly glycolysis, nutrient transport, and cellular respiration are inhibited during fluoride exposure, presumably through metalloprotein inhibition (Feig *et al.* 1971; Fina *et al.* 2014; Rogalska *et al.* 2017).

One of the most cited protein targets for fluoride inhibition is enolase. This enzyme catalyzes the penultimate step of glycolysis, converting 3-phosphoglycerate to phosphoenolpyruvate. Fluoride's ability to bind enolase was discovered accidentally by Warburg and Christian (Warburg and Christian 1942). Their lab in Germany happened to use water high in fluoride while working to crystallize enolase, and during structural analysis they found fluoride bound to phosphate and magnesium at enolase's active site. Later enzymatic analysis found that enolase has one of the lowest  $K_D$ 's for fluoride, at 1-10 mM (20-200 ppm) depending on species (Cimasoni 1972; Wang and Himoe 1974, Shahed *et al.* 1980; Maurer and Nowak 1981; Kaufmann and Bartholmes 1992; Qin *et al.* 2006). Although enolase inhibition is often referenced as fluoride's main mechanism of toxicity, several papers have offered data against this hypothesis. Fluoride-resistant species of bacteria had no significant change in enolase activity or sequence compared to wild type (van Loveren *et al.* 1990, 1991, 2008; Mitsuhashi *et al.* 2014; Liao *et al.* 2015 and 2016). RNA-Seq analyses of mammalian and

plant cells exposed to fluoride reported no changes in enolase expression (Li *et al.* 2017; Pereira *et al.* 2018). Overexpression of enolase in fluoride-sensitized yeast produced no change in fluoride resistance (Johnston and Strobel 2019). Clinical researchers, using fluoride to inhibit glucose consumption in stored blood, found that metabolic activity decreased only after the induction of stress signaling from high fluoride (Montagnana and Lippi 2017). One potential explanation is that metabolism is inhibited during fluoride exposure as a side effect of stress induction, such as oxidative stress or intracellular acidification. Apoptotic signaling, oxidative stress, and intracellular acidification each have been reported to inhibit glycolysis (Halperin *et al.* 1968, Corretti *et al.* 1991, Erecinska *et al.* 1995, Souza *et al.* 2002, Bensaad *et al.* 2006, Shi *et al.* 2009, Hegde *et al.* 2010, Pradelli *et al.* 2014). Several studies found that acidification alone reduced glycolysis to a greater degree than fluoride treatment (Boink *et al.* 1994; Belli *et al.* 1995; Gambino *et al.* 2009). Regardless of mechanism, glycolysis is consistently inhibited across organism models during fluoride toxicity.

#### Inhibition of proteins by metallo-fluoride substrate mimics

Fluoride toxicity is greatly enhanced when complexed with metal. Among the most toxic (and most studied) complexes are aluminum- ( $\text{AlF}_{3,4}$ ) and beryllium-fluoride ( $\text{BeF}_{3,4}$ ). These compounds are isomorphous to phosphate, and consequently able to inhibit phosphoryl-transfer enzymes (Chabre 1990; Petsko 2000). Aluminum- and beryllium-fluoride are effective phosphate mimics due to their radius of 1.55 Å (phosphate is 1.60 Å); 28 other metals tested in complex with fluoride do not act as phosphate mimics (Martin 1986; Li 2003). Nonetheless, the affinity for each metallo-fluoride to various enzymes depends on the pH and interaction with essential positively-charged amino acids (Schlichting and Reinstein 1999; Strunecka *et al.* 2002). Over 100 enzymes have been crystallized with aluminum- or beryllium-fluoride, the majority of which are classified as either ATPases, GTPases, or kinases (Berman *et al.* 2000).

Aluminum- and beryllium-fluoride can also alter the phosphorylation state of various proteins, particularly through GTP mimicry. Many proteins, including G-proteins, actin, and microtubules, are regulated by GTP binding; GDP-bound G-proteins are in an “off” state, while GTP-bound proteins are “on.” In the case of aluminum- or beryllium-fluoride exposure, both metallo-fluorides bind GDP to mimic the bound  $\gamma$ -phosphate of GTP (Bigay *et al.* 1987; Higashijima *et al.* 1987; Antony and Chabre 1991).  $\text{AlF}_x$  and  $\text{BeF}_x$  also stabilize the transition state for the “on” conformation. Consequently, proteins activated by metallo-fluoride are more stable than those activated naturally, and stay in the “on” conformation (Li 2003).

Because of the far-reaching roles of GTPase regulation, their non-selective activation by metallo-fluorides leads to wide dysregulation of functions including cell signaling, transport, and cytoskeleton integrity (Combeau and Cartier 1989; Muhlrud *et al.* 1994; Allen *et al.* 1996, Loweth *et al.* 1996; Li 2003; Agalakova and Gusev 2011; Park *et al.* 2013).

Free fluoride can also activate protein and pathway activity by altering phosphorylation states. Rho GTPase-binding proteins have been shown to bind to GTPases and stabilize the transition state in a fluoride, but not aluminum-dependent manner (Yatani and Brown 1991; Antonny *et al.* 1993; Vincent *et al.* 1998). Instead, in both cases magnesium was found to be essential, and could theoretically form a magnesium-fluoride phosphate mimic.

#### Activation of proteins by fluoride

While high levels of fluoride inhibit a wide variety of proteins, low levels of fluoride have been reported to activate certain enzymes through unknown mechanisms. *In vivo*, fluoride activates glucose-6-phosphate dehydrogenase, 5'-nucleotidase,  $\alpha$ -amylase,  $\beta$ -fructofuranosidase, creatine kinase, and phosphodiesterase I (Adamek *et al.* 2005). These enzymes are all metalloproteins, and the total activity per enzyme is increased in the presence of fluoride. Other enzymes, such as alkaline phosphatases, aminotransferases, and lactate dehydrogenases, are shown to either increase or decrease in activity upon fluoride exposure, depending on report and tissue type (Bogin *et al.* 1976; Singh and Kanwar 1981; Singh 1984; Nakade *et al.* 1999; Adamek *et al.* 2005). However, this activation is thought to be more stress-induced than from direct stimulation of the enzymes.

#### **V. Fluoride-induced pH and electrolyte imbalance**

At both the single- and multi-cellular level, fluoride exposure triggers acidification and electrolyte imbalance. The exact cause is unknown. Prolonged exposure of vertebrates to high fluoride results in the loss of calcium and magnesium from the plasma, and an excess of potassium (Dalamaga *et al.* 2008). Complementary to this finding, fluoride exposure in single cells results in an influx of calcium and magnesium, and a loss of potassium (Johnston and Strobel 2019). This effect has been proposed to be due to either downstream stress signaling, or the binding of fluoride to metals (Boink *et al.* 1994; Giachini and Pierleoni 2004). Regardless of mechanism, the imbalance of electrolytes in organisms from fluoride exposure has far reaching implication in cell homeostasis and signaling disruption.

Fluoride exposure is also associated with a drop in intracellular pH. Fluoride is a weak acid that enters cells as HF and dissociates, thus releasing one proton per fluoride. Consequently, the more fluoride that enters a cell, the more acidic that cell becomes. However, fluoride causes intracellular acidification to a larger degree than can be explained by proton shuttling (Kawase and Suzuki 1989; Hamilton 1990; Belli *et al.* 1995; Marquis 1995; Guha-Chowdhury *et al.* 1996; Marquis *et al.* 2003; Gassowska *et al.* 2013). Many hypotheses have been put forward to explain this observation. Among these, the most common are metabolic disruption, perturbation of the mitochondria, transmembrane protein inhibition, and induction of stress signaling.

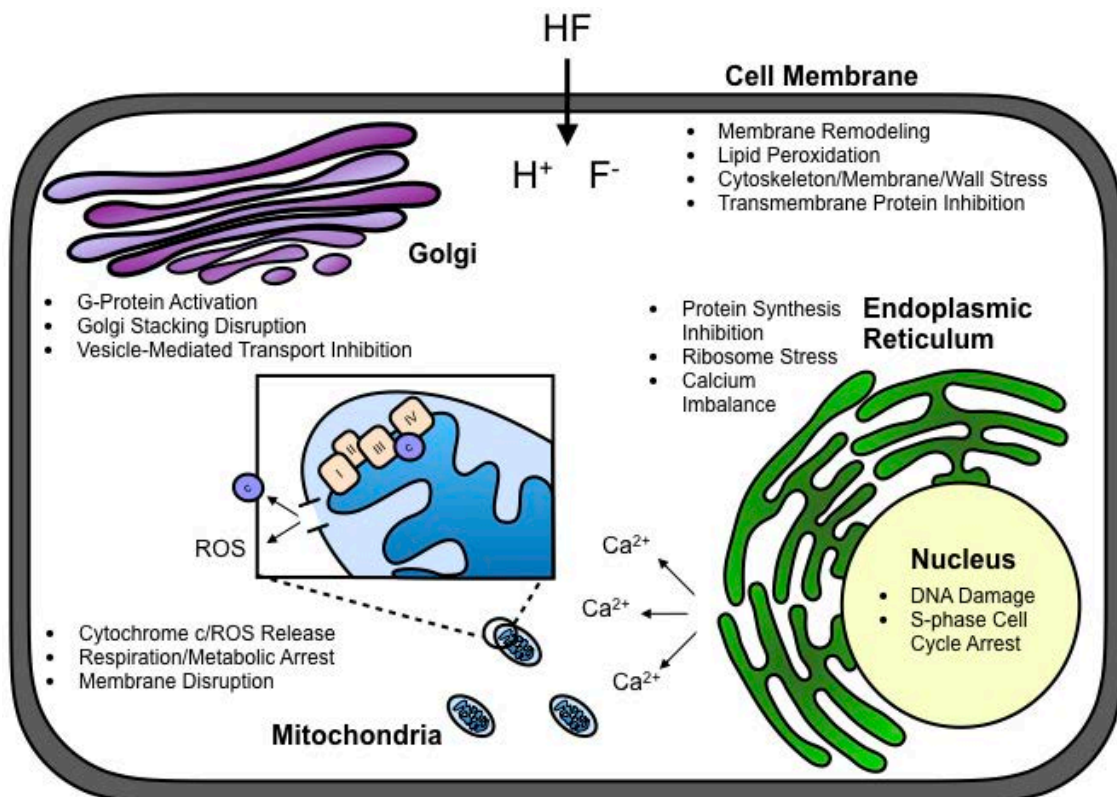
Fluoride inhibits metabolism through an unclear mechanism, the downstream effects of which are the reduction in intracellular ATP and damage to the mitochondria. ATP is reduced both in cells containing mitochondria – which display signs of permanent damage and reduced respiration after fluoride exposure – and in erythrocytes (red blood cells), which lack mitochondria and produce ATP solely through anaerobic glycolysis (Feig *et al.* 1971; Jeng *et al.* 1998, Agalakova and Gusev 2012; Fina *et al.* 2014). ATP depletion leads to the hydrolysis of ATP into ADP and AMP along with the release of protons, consequently leading to intracellular acidification. Damage to the mitochondria releases free radicals, resulting in oxidative stress (Kowaltowski *et al.* 2001; Chen *et al.* 2003; Batandier *et al.* 2004; Gogvadze *et al.* 2006). This in turn causes DNA damage, metabolic disruption, ATP hydrolysis, protein inhibition, and intracellular acidification (Boonstra and Post 2004; Sharma *et al.* 2012; Fu *et al.* 2014). However, just as free radicals are known to disrupt metabolism, acidify the cell, and activate stress signaling, each of these phenotypes also activate the release of free radicals from the mitochondria (Liu *et al.* 2003; Berezhnov *et al.* 2016). Similarly, high fluoride induces cell cycle arrest and apoptotic signaling, which again triggers the release of free radicals (Zhang *et al.* 2008). As such, the sequence of events between each reported phenomenon are difficult to determine.

Another potential explanation for the drop in pH is fluoride inhibiting transmembrane proton transporters. Key among them are the Na<sup>+</sup>/H<sup>+</sup> symporters, ATPases, and G-coupled proteins. Fluoride generally inhibits transmembrane proteins by changing the protein's conformation to its transitional state, such as metallo-fluoride forcing Na<sup>+</sup>/K<sup>+</sup>-ATPase into its E2P state (Swann 1990; Murphy and Hoover 1992; Gassowska *et al.* 2013; Montes *et al.* 2015). Transmembrane proteins are essential for maintaining the plasma membrane potential of cells. Disruption of the membrane electrochemical gradient disrupts a wide range of cellular

processes, and could potentially shift intracellular pH. In the case of  $\text{Na}^+/\text{H}^+$  symporter inhibition, a function antiporter exports  $\text{Na}^+$  from the cell while importing  $\text{H}^+$ . As such, the inhibition of the protein by fluoride would theoretically accumulate extracellular protons. However, the opposite is shown to occur (Kawase and Suzuki 1989). Kawase and Suzuki proposed that this extracellular proton buildup would result in increased fluoride protonation, which would therefore increase overall fluoride toxicity and stress signaling. It also could be case that while this particular protein inhibition raises extracellular pH, the overall inhibition of transmembrane transporters leads to a net accumulation of protons.

## VI. Organelle disruption by fluoride

Prolonged exposure to high levels of fluoride leads to widespread organelle damage. This damage is both time and concentration dependent. While the sensitivity of each organelle to fluoride varies slightly by organism, in general, fluoride disrupts the cell surface, mitochondria, endoplasmic reticulum, Golgi, and nucleus (Fig. 3).



**Figure 3: Intracellular fluoride toxicity.** General scheme of downstream organelle damage after prolonged exposure to high fluoride, conserved across eukaryotes.



Fluoride can trigger irreversible damage to the cell surface. Organisms that have calcium-pectate in their cell walls, such as plants, are prone to calcium depletion upon fluoride exposure (Tsunoda and Yu 1985). The plasma membrane is prone to massive remodeling. Among the processes induced are peptidoglycan turnover, actin production, reduced membrane functions, decreased cell adherence, and cytoskeleton rearrangement (Leshner *et al.* 1977; Ben-Hur *et al.* 1992; Li *et al.* 2005; Mendoza-Schulz *et al.* 2009; Liang *et al.* 2015; Tsaplina and Khaitlina 2016; Chen *et al.* 2017; He *et al.* 2018). In addition, fluoride causes widespread lipid peroxidation to the plasma membrane (Shivarajashankara *et al.* 2001; Shanthakumari *et al.* 2004; Wang *et al.* 2004). These effects are not specific to fluoride; many stressors are known to induce membrane remodeling (Mollapour *et al.* 2006; Antonelou *et al.* 2011; Farah *et al.* 2011; Brandao *et al.* 2014; Guo *et al.* 2018; Westman *et al.* 2019). While most membrane changes are attributed to either oxidative stress, apoptotic signaling or lipid peroxidation, free or metallo-fluoride can also directly bind actin and change its polymerization (Combeau and Carlier 1989; Allen *et al.* 1996; Li *et al.* 2005; Kim *et al.* 2007; He *et al.* 2018). Regardless of membrane damage, cell cycle arrest during fluoride exposure is enhanced with an increased uptake in fluoride. This suggests that fluoride's principal mechanism of toxicity at high doses is intracellular (Li and Breaker 2012; Ji *et al.* 2014).

The most widely reported fluoride target is the mitochondria. Many of the mitochondrial proteins are metalloproteins, and have been linked to fluoride inhibition (Batenburg and van den Bergh 1972; Muijsers *et al.* 1974; Lavrushenko 1978). Among these are the respiratory complexes I-IV, and F-ATPase (Sutton *et al.* 1987; Fina *et al.* 2014; Zhao *et al.* 2019). Either directly or indirectly, fluoride exposure damages mitochondrial membrane integrity (Izquierdo-Vega *et al.* 2008; Yang *et al.* 2014; Yan *et al.* 2015). This reduces the overall activity of the mitochondria, inhibits cellular respiration, and triggers mitochondrial leakage (Evans 1922; Miller and Miller 1974; Jothiramajayam *et al.* 2014). Most notably, fluoride causes leakage of free radicals and cytochrome c into the cytoplasm, thus triggering oxidative stress (Anuradha *et al.* 2001; Lee *et al.* 2008). Leakage of free radicals into the cytoplasm is particularly problematic, as fluoride is also thought to inhibit proteins that degrade free radicals, such as superoxide dismutase and glutathione peroxidase (Lawson and Yu 2003; Inkielewicz-Stepniak and Krechniak 2004; Mittal and Flora 2006; Hassan and Yousef 2009). The release of free radicals and concurrent loss of mitochondrial activity following

fluoride exposure has been widely reported across species and tissue types (Vani *et al.* 2000; Tao *et al.* 2004; Jin *et al.* 2007; Barbier *et al.* 2010; Li *et al.* 2012; Jothiramajayam *et al.* 2014). As such, the addition of antioxidants partially rescues from fluoride toxicity (Basha and Sujitha 2011; Nabavi *et al.* 2012; Vasant and Narasimhacharya 2012; Cao *et al.* 2015; Basha *et al.* 2017). The last few years has seen renewed interest in mitochondrial inactivation under fluoride exposure as the primary mechanism of fluoride toxicity, as much of the known adverse effects can be attributed to free radicals (Farrugia and Balzan 2012; Phaniendra *et al.* 2015; Quijano *et al.* 2016).

Presumably through free radical oxidation, fluoride-exposed cells undergo intensive DNA damage. Fluoride triggers both single- and double-stranded DNA damage following oxidative stress (Flora *et al.* 2012; Podder *et al.* 2015; Susik *et al.* 2015; Zhang *et al.* 2016; Deng *et al.* 2017). Typically, studies that report DNA damage expose mice or rats to high concentrations of fluoride for weeks to months. The necessity of a long incubation time suggests that DNA damage is a downstream stress effect rather than a direct target of fluoride. This supports the hypothesis that DNA damage is a result of oxidative stress, rather than a phenotype specific to fluoride. Due to DNA damage, high fluoride eventually leads to S-phase cell cycle arrest (Hayashi and Tsutsui 1993; Wang *et al.* 2004; He and Chen 2006; Zhang *et al.* 2008; Su *et al.* 2016; Liu *et al.* 2018).

The endoplasmic reticulum (E.R.) undergoes organelle stress, calcium imbalance, and unfolded protein stress under fluoride exposure. The E.R. has many functions, including protein synthesis, detoxification of compounds, and calcium storage (Schwarz and Blower 2016; Pollard *et al.* 2017). Disruption of E.R. activity leads to an accumulation of unfolded proteins, which trigger the unfolded protein response (UPR) pathway through either IRE1, PERK, or ATF6 activation (Liu and Kaufman 2003; Sharma *et al.* 2008; Wolfson *et al.* 2008). Prolonged exposure to fluoride gives way to structural E.R. damage (Matsuo *et al.* 2000; Yanni 2000; Quadri *et al.* 2018a). This has been shown to elicit E.R. stress, as well as activation of the UPR pathway through IRE1 and PERK (Kubota *et al.* 2005; Sharma *et al.* 2008; Xu *et al.* 2010; Liu *et al.* 2016). ATF6 is also activated, although several labs saw less activation of ATF6 compared with IRE1 and PERK (Ito *et al.* 2009; Xu *et al.* 2010; Zhang *et al.* 2013).

The exact mechanism by which fluoride causes E.R. stress is unclear. Prolonged fluoride exposure inactivates protein synthesis (Matsuo *et al.* 2000; Wei *et al.* 2011). Widespread protein synthesis inhibition would lead to E.R. stress and could partially account for the mechanism. However, the UPR does not

sufficiently explain the extent of E.R. damage upon fluoride exposure. Fluoride, either directly or indirectly, disrupts  $\text{Ca}^{2+}$  homeostasis (Borke and Whitford 1999; Zhang *et al.* 2016; Wang *et al.* 2018). Fluoride is known to coordinate with metals, and could scavenge  $\text{Ca}^{2+}$  from the E.R. Metallo-fluoride can target  $\text{Ca}^{2+}$ -ATPase, which is essential for  $\text{Ca}^{2+}$  uptake (Coll and Murphy 1992; Murphy and Coll 1992; Hawkins *et al.* 1994; Xu *et al.* 2007). Disruption of this ATPase could lead to E.R. stress and a release of  $\text{Ca}^{2+}$  into the cytoplasm (MacLennan *et al.* 1997; Mekahli *et al.* 2011). Furthermore,  $\text{Ca}^{2+}$  is a signaling molecule for the mitochondrial oxidative stress pathway, its release into the cytoplasm activates the release of free radicals (Ermak and Davies 2002; Gorlach *et al.* 2015). UPR induction requires further ATP provided by the mitochondria, which again could trigger the release of free radicals (Suzuki *et al.* 2014). In support of the hypothesis that E.R. stress triggers oxidative stress, the addition of E.R. stress inhibitors reduced DNA damage (Kubota *et al.* 2005; Deng *et al.* 2016). Despite this, two labs found that the addition of antioxidants reduced E.R. stress; this suggests that either free radical release are upstream of, or exacerbate E.R. stress (Suzuki *et al.* 2014; Yang *et al.* 2014b; 2015). Regardless of mechanism, E.R. activity is disrupted during fluoride exposure.

Not much is known about fluoride's effect on the vacuole. The intracellular distribution of fluoride is unknown, although hypothesized to predominate in the cytoplasm of most cells. However, a study by the Mao lab on fluoride-accumulating tea leaves found that 98% of total fluoride was found in the vacuoles (Gao *et al.* 2014). While this may seem unexpected, the authors noted that fluoride uptake correlated with aluminum uptake. Aluminum complexes with fluoride, and like all metals, is transported to the vacuole for storage. Free fluoride has been shown to reduce V-ATPase activity, a proton transporter that is essential for vacuolar function (Baunthiyal and Sharma 2014). Furthermore, rats exposed to 17 mg/kg body weight fluoride for 4 days displayed signs of deformed, inactive vacuoles in their ameloblast cells (Walton and Eisenmann 1974). Nonetheless, very few fluoride studies have reported a change in vacuolar activity upon fluoride exposure.

Fluoride inhibits Golgi stacking, although this effect is reversible. Aluminum-fluoride ( $\text{AlF}_4^-$ ) has been shown to activate G-proteins by mimicking the gamma-phosphate of GTP (Bigay *et al.* 1985; Coleman *et al.* 1994; de Boer *et al.* 1994; Mukhopadhyay *et al.* 1997). As G-proteins function in signal transduction, their activation has far-reaching implications, especially in vesicle-mediated exocytosis and Golgi function.

Activation of plasma membrane G-proteins by aluminum-fluoride leads to a calcium-dependent activation of exocytosis (Elferink *et al.* 1980; Elferink and Deierauf 1989; Komatsu *et al.* 1994). G-proteins are also found on the Golgi, and are essential for Golgi stacking (Jamora *et al.* 1997; van Hook 2015). The Rothman lab demonstrated that addition of aluminum-fluoride to a cell-free system inhibited protein transport between Golgi stacks (Melancon *et al.* 1987). Aluminum-fluoride also alters the protein coating assembly along the Golgi (Finazzi *et al.* 1994; Tomas *et al.* 2010). Later studies in neuroendocrine cells found that free fluoride also inhibited Golgi stacking by interfering with matrix and cisternae protein assembly, and that this effect was reversed within two hours of recovery (Back *et al.* 2004). However, the fluoride may still have been in a metal complex; a study by the Lowe lab demonstrated that in the absence of aluminum, fluoride will combine with magnesium to bind g-proteins (Graham *et al.* 1999). As a whole, fluoride reversibly inhibits Golgi function, although this might only happen when fluoride is complexed with metal.

Fluoride exposure inhibits protein synthesis, an effect linked to both stress-signaling and ribosome inhibition. Stress pathways, particularly oxidative stress, are known to inhibit protein synthesis (Sheikh and Fornace 1999; Sonenberg and Hinnebusch 2009; Liu and Qian 2014). This effect also occurs during ATP depletion, a well-established fluoride effect (Freudenberg and Mager 1971). Unsurprisingly, fluoride exposure has been shown to reduce the turnover of new proteins (Hongslo and Holland 1979; Sharma *et al.* 2008). However, its mechanism is thought to be independent of both stress-signaling and ATP depletion. Early studies on reticulocytes and the mold *D. discoideum* found that fluoride causes reversible inhibition of polyribosome formation (Bishop 1967; Colombo *et al.* 1968; Hogan 1969; Lubsen and Davis 1972; Sameshima *et al.* 1972; Godchaux III and Atwood IV 1976). Instead, fluoride leads to a formation coined “NaF ribosomes” – ribosomes believed to either contain an extra 40S subunit, or in some cases accumulate deacetylated tRNA<sup>Met</sup> bound to the 40S complex (making a 43S subunit) (Hoerz and McCarty 1969; Culp *et al.* 1970; Vesco and Colombo 1970; Geraghty *et al.* 1973; O’Rourke and Godchaux III 1975; Holland 1979). Overall, the formation of this complex results in inhibition of polypeptide chain initiation (Lin *et al.* 1966; Ravel *et al.* 1966; Lebleu *et al.* 1967; Mosteller *et al.* 1967). This inhibition occurs after edeine inhibition (which prevents tRNA binding to the P site), and before cycloheximide inhibition (which prevents translational elongation) (Obrig *et al.* 1970; 1971; Hardesty *et al.* 1973; Dinos *et al.* 2004). The research on this effect was conducted in the 1960-1970s; nowadays, the direct action of fluoride on ribosomes has been

largely forgotten. Fluoride has been widely recorded to halt protein translation, but this effect is generally linked to apoptotic signaling. Recent RNA-Seq data have reported strongly repressed ribosomal subunit expression during fluoride exposure, indicating that this underappreciated phenotype may play a major role in fluoride toxicity (de Souza Melo *et al.* 2017; Li *et al.* 2017; 2018; Johnston and Strobel 2019).

## **VII. Fluoride exposure in vertebrates**

Mammals exposed to high doses of fluoride experience widespread tissue toxicity. The target of fluoride depends both on how the fluoride is administered and in what context. Typically, fluoride is either inhaled or ingested. Fluoride present in beverages or food passes along the gastrointestinal tract. Fluoride readily passes through biological membranes as hydrofluoric acid (HF). As such, exposure to fluoride as HF leads to widespread tissue damage. Exposure to fluoride as NaF or KF dissociates and travels along the gastrointestinal tract and plasma, and can accumulate in soft tissue such as the spleen, kidney, and bone.

### Lung/Epithelial Toxicity

The most toxic form of fluoride is HF. HF, commonly released as industrial or volcanic fumes, vaporizes above 20°C and so is normally found as a gas. Low concentrations of HF cause eye and respiratory irritation. Volunteers experienced nasal irritation after 1 minute of exposure to 32 ppm, and after 8 hours of exposure to 4.6 ppm HF (Machle *et al.* 1934; Collings *et al.* 1951). Prolonged exposure to HF causes electrolyte imbalance, including hypocalcemia (McKee *et al.* 2014). Namely, HF causes an excess of potassium and deficiency of calcium and magnesium in the plasma. HF is corrosive, and high concentrations cause deep tissue damage, nerve damage, pulmonary edema and cardiac arrhythmia (NRC 2004). Inhalation of 50-250 ppm over a five-minute interval has caused lethality (Henderson and Haggard 1943; Halton *et al.* 1984). Because HF is readily absorbed by skin, organisms that come into contact with liquid HF suffer from heavy burns. Exposure of 2.5% of total skin area with 70% HF can be fatal (Tepperman 1980). Treatment for HF exposure involves ingesting calcium pills to scavenge fluoride. However, because of HF's ability to rapidly pass through cellular membranes, autopsies frequently show fluoride poisoning in tissue far from the original point of fluoride contact (Sheridan *et al.* 1995; Cordero *et al.* 2004). While HF toxicity is a problem in industrial settings, the general population is unlikely to encounter high levels of HF. Instead, most people encounter free fluoride in their food, water, and oral products.

### Dental Fluorosis

An excess of fluoride exposure leads to detrimental tooth toxicity. Fluorapatite is mechanically weaker than hydroxyapatite, and too much fluorapatite leads to brittle and mottled teeth in the condition known as dental fluorosis (Thylstrup and Fejerskov 1978; Giambro *et al.* 1995; den Besten and Li 2011). Dental fluorosis occurs in healthy individuals exposed to higher than 1.5 ppm (75  $\mu\text{M}$ ) fluoride, although malnourished children in India displayed signs of fluorosis at 0.5 ppm (25  $\mu\text{M}$ ) fluoride exposure (Ayoob and Gupta 2006). A study comparing the incidence of fluorosis in U.S. adolescents from the years 1986-1987 and 1999-2004 found that fluorosis increased from 22.6% to 40.7% of adolescents surveyed (Beltran-Aguilar *et al.* 2010). Fluoride most readily forms fluorapatite as fresh enamel is developing; as such, mammals are most sensitive to dental fluorosis during tooth development. For humans, this means that juveniles up to 8 years of age (with particular sensitivity during the first 2 years) are susceptible to fluorosis (Bardsen and Bjorvatn 1998; Bardsen 1999; Hong *et al.* 2006; Bhagavatula *et al.* 2016). Many studies on fluorosis use rodents, because rodents develop incisors throughout their life and are constantly producing enamel (Warshawsky and Smith 1974; Angmar-Mansson and Whitford 1982). Although the practice of water fluoridation is implemented to promote dental health, excess fluoride passes through the gastrointestinal tract and can cause additional toxicity at high concentrations.

### Ameloblast Toxicity

Any fluoride present in the mouth that does not react with enamel can cause toxicity to ameloblasts (cells that function in tooth development). Although it is well established that fluoride causes fluorapatite formation in teeth and that excess fluoride leads to fluorosis, the exact mechanism of dental fluorosis has never been shown. Several mechanisms besides remineralization have been put forward, most notably that fluorosis is caused by ameloblast damage (Kruger 1970; Walton and Eisenmann 1974; Fejerskov *et al.* 1977). In the Kruger and Eisenmann studies, exposure of rats to 0.1, 3, 7, and 17 mg F/kg body weight resulted in damage to the vacuoles, mitochondria, and endoplasmic reticulum of ameloblasts. Later studies found that the rate of mineral deposition and maturation was dramatically reduced in the ameloblasts of rats upon exposure to 5-150 ppm NaF, presumably because of intracellular damage (den Besten *et al.* 1985; Pergolizzi *et al.* 1995; Bronckers *et al.* 2009; Wei *et al.* 2016). Fluoride exposure to ameloblasts in cell culture causes endoplasmic reticulum stress, DNA fragmentation, cytoskeleton defects, protein synthesis inhibition, reduced secretion of

enamel matrix proteins, and eventual apoptosis (Kubota *et al.* 2005; Li *et al.* 2005, Hassanuma *et al.* 2007; Wang *et al.* 2016). However, it is important to note that these studies are typically conducted at much higher fluoride (100-150 ppm) than levels causing dental fluorosis in humans (>1.5 ppm).

### Skeletal Fluorosis

Skeletal fluorosis is the most severe form of chronic fluoride toxicity. The underlying mechanics of skeletal fluorosis are similar to dental fluorosis: high fluoride exposure leads to a change in mineral confirmation, as well as stress to surrounding cells. Ingested fluoride gradually builds up in tissue such as the spleen and kidney, but over 90% of accumulated fluoride associates with the bone (Savchuck and Armstrong 1951; Sharma *et al.* 1977; Rao *et al.* 1995; Palczewska-Komsa *et al.* 2014). The high affinity of bones for fluoride is most likely due to their high calcium content. *In vitro* soaking of mouse femurs with 1.5 M (28,000 ppm) fluoride resulted in 30-fold uptake of fluoride within 12 hours (Silva and Ulrich 2000). The uptake of fluoride into bone results in the conversion of the bone mineral hydroxyapatite into fluorapatite, which alters the general bone lattice and reduces its overall strength (Grynypas 1990; Grynypas *et al.* 1992; Waddington and Langley 2003; Mousny *et al.* 2008). As such, studies involving soaking bones in molar concentrations of fluoride found that the bones were mechanically weaker than normal (Walsh and Guzelsu 1993; Abjornson *et al.* 1998; Silva and Ulrich 2000; DePaula *et al.* 2002). Similarly, vertebrates exposed to high fluoride have mechanically weaker bones (Evans and Wood 1976; Sogaard *et al.* 1995; Turner *et al.* 1995; Bohatyrewicz 1999). This increased brittleness is associated with skeletal dysmorphia and an increased risk of fracture.

The onset of skeletal fluorosis in humans varies by individual. The reported dosage required for developing skeletal fluorosis varies widely, and is affected by age, metabolic rate, genetic disposition, and overall health of the individual (Marier *et al.* 1963; Krishnamachari 1986; Wang *et al.* 1994; U. S. DHHS 1991; ATSDR 1993; Pramanik and Saha 2017). As the kidneys are essential for filtering fluoride, patients with kidney disorders are also at high risk for skeletal fluorosis (Linsman and McMurray 1943; Call *et al.* 1965; Gerster *et al.* 1983; Fisher *et al.* 1989; Bansal and Tiwari 2006). It is estimated that for the average person, 6-10 mg per day of fluoride ingestion for at least 10 years leads to skeletal fluorosis (Whitford 1996; ATSDR 2003; Kurdi 2016). Children are the most susceptible, and accumulate fluoride at a greatly accelerated rate compared with adults (Teotia *et al.* 1998; Patel *et al.* 2017; Shruthi and Anil 2018). Symptoms of skeletal fluorosis vary by individual and degree of fluoride intake. For some, exposure to >6

ppm fluoride in over 10 years of adulthood resulted in no skeletal defects, only arthritic and gastric pain (Cook 1971; Czerwinski *et al.* 1988). As the most severe case, patients exposed to high fluoride during development are predisposed to skeletal bowing, rickets, and fractures, along with combinations of osteomalacia, osteoporosis, and osteosclerosis (Lemmon 1934; Blue 1938; Christie 1980; Krishnamachari 1986).

Skeletal fluorosis causes stress to surrounding osteoblasts. Low fluoride concentrations (5-1000  $\mu\text{M}$ , or 0.1-200 ppm *in vitro*), stimulates osteoblasts (Hall 1987; Bellows *et al.* 1990; 1993; Modrowski *et al.* 1992; Lau and Baylink 1998; Qu *et al.* 2008). The exact mechanism by which fluoride causes osteoblast proliferation is unclear, although it has been shown that fluoride causes an uptake in phosphate, increased alkaline phosphatase activity, decreased acid phosphatase activity, and an activation of the MAPK pathway (Farley *et al.* 1983; 1988; Lau *et al.* 1989; Khokher and Dandona 1990; Selz *et al.* 1991; Burgener *et al.* 1995; Susa 1999; Lau *et al.* 2002; Lee *et al.* 2017). The stimulation of osteoblasts triggers new bone formation and an increase in overall bone density in patients with over 0.2% fluoride in bones (Aaron *et al.* 1991). However, the bone matrix is disrupted by fluoride, and the improved bone mass is of lower quality and increases the risk of fractures (Carter and Beaupre 1990; Aaron *et al.* 1991; Hillier *et al.* 1996). Fluoride above 1 mM (20 ppm) results in osteoblast cytotoxicity, particularly to the nucleus and endoplasmic reticulum (Bellows *et al.* 1990; Qu *et al.* 2008; Zhou *et al.* 2013; Liu *et al.* 2014; Shenoy *et al.* 2019). As such, there is an overall bimodal trend of low fluoride stimulating bone mass, while high fluoride decreases bone mass.

### Gastrointestinal Toxicity

From the mouth, fluoride passes through the esophagus and into the stomach. Acute fluoride toxicity in the stomach occurs after ingestion of 0.1-8 mg/kg bodyweight (Akiniwa 1997; Shulman and Wells 2007; Ullah *et al.* 2017). This would be equivalent to approximately 7.5-56 grams of toothpaste (10-50% of the tube), or 7.5-56 liters of 1 ppm fluoridated water for a 75 kg adult. In areas with high fluoride in the groundwater such Kerala, India (5.8 ppm), a 75 kg adult would need to drink much less water (1.3 L) to experience acute gastrointestinal toxicity (Shaji *et al.* 2007). Symptoms of gastrointestinal toxicity include nausea, diarrhea, headaches, and gastric pain (Das *et al.* 1994).



Fluoride's main mechanism of distribution relies on forming HF and passing through cell and tissue membranes. Any cell or tissue is more sensitive to fluoride in an acidic environment (Gutknecht and Walter 1981; Whitford and Pashley 1984; Ji *et al.* 2014). The mouth, esophagus, and upper stomach are at neutral pH (6.5-7.5, 5.0-7.0 and 4.0-6.5, respectively) (Fallingborg 1999; Tutuian and Castell 2006). However, the lower stomach is highly acidic (pH 1.5-3.5), and fluoride readily forms HF. Fluoride has a pKa of 3.2. This means in the lower stomach, 67-96% of fluoride is in HF form and can dissociate in equilibrium across the plasma membranes of cells. Nonetheless, only 25% of ingested fluoride is thought to be absorbed by the stomach and pass on to other cells; the rest of fluoride is moved to the intestines (Kanduti *et al.* 2016). High exposure to fluoride can damage the stomach lining, but this effect is not seen with chronic exposure to low fluoride (Pashley *et al.* 1984; Susheela and Das 1988; de Oliveira *et al.* 2017).

The majority of ingested fluoride is absorbed by the small intestine. Only ~10% of consumed fluoride is never absorbed by the body and excreted through feces (Whitford 1994; Buzalaf and Whitford 2011). Fluoride has been hypothesized to pass through the small intestines as its anion form F<sup>-</sup> (Nopakun and Messer 1990). Because of fluoride's reactivity, the nutrients ingested along with fluoride, especially those rich in calcium, influence the amount of fluoride absorbed by the intestines (Whitford 1994; Cury *et al.* 2005). High levels of fluoride lead to a large array of stress effects in the small intestine, including inflammation, oxidative stress, and tissue damage (Butler *et al.* 1990; Chauhan *et al.* 2013; de la Fuente *et al.* 2016; Wang *et al.* 2018). Chronic exposure to high fluoride has been correlated with inflammatory bowel disease and nerve damage along the gastrointestinal tract (Dasarathy *et al.* 1995; Follin-Arbelet and Moum 2016; de Souza Melo *et al.* 2017). In rats, prolonged exposure to ≥10 mM NaF (190 ppm) led to permanent damage in mucosal cell membranes (Easmann *et al.* 1984; Pashley *et al.* 1984). Further investigations into mucosal cell damage shows alterations to the mitochondria, inhibition of protein synthesis, metabolic arrest, disruption of cytokine production, and an upregulation of genes for scavenging nutrients (Jeng *et al.* 1998; Chauhan *et al.* 2013; Luo *et al.* 2013; Dionizio *et al.* 2018). However, just as in the cases of stomach and ameloblast toxicity, these studies were conducted at much higher levels than what organisms would be exposed to from most naturally occurring groundwaters.

### Plasma/Kidney/Liver Toxicity

Approximately 30-60 minutes after ingestion, fluoride is absorbed by the small intestine and reaches peak concentrations in the bloodstream (Nopakun *et al.* 1989; Ponikvar 2008). Erythrocytes (red blood cells) lack organelles, and high fluoride exposure causes metabolic arrest and membrane damage (Feig *et al.* 1971; Susheela and Jain 1986; Agalakova and Gusev 2013). Rats given 30-100 mg/kg body weight fluoride showed widespread cardiovascular toxicity, including oxidative stress throughout the bloodstream (Shivarajashankara *et al.* 2003; Panneerselvam *et al.* 2015). Plasma with high fluoride generally has low calcium and magnesium and high potassium, which is believed to be caused by both fluoride scavenging metals and disruption of potassium channels (Baltazar *et al.* 1980; Cummings and McIver 1988; Boink *et al.* 1994; Su *et al.* 2003). Both oxidative stress and ion imbalance leads to cardiac arrest, and ingestion of >15 mg/kg bodyweight fluoride can lead to cardiac irregularities (Leone *et al.* 1956; Karpova 1989; Ullah *et al.* 2017). However, cardiac arrest from fluoride has only been reported in cases where individuals are exposed to very high levels of fluoride, and especially in its acid form. As an ion, fluoride generally is filtered from the bloodstream by the kidney or accumulates in soft tissue without causing widespread plasma stress.

The kidney is one of the most sensitive tissues to fluoride, second only to bone. Because the kidney filters blood, the kidney is exposed to five times higher plasma, and therefore five times higher fluoride (Whitford 1996). Approximately 50% of free fluoride is filtered by the kidney (Whitford 1994b; Marier and Rose 1977). Exposure to fluoride at normal fluoridation levels (0.7-1.5 ppm) shows no effect on the kidney; kidney damage occurs after 50-80  $\mu\text{M}$  fluoride in the serum (Cousins *et al.* 1986; Partanen 2002; Ludlow *et al.* 2007; Wasana *et al.* 2015). For reasons not quite understood, children are more susceptible to kidney damage from fluoride than adults (Ekstrand *et al.* 1994; Dharmaratne 2015). Fluoride damages the nucleus, mitochondria, vacuole, endoplasmic reticulum, and cell membrane of kidneys in rats, rabbits, and humans exposed to 1-5 mM NaF (20-100 ppm) (Shashi *et al.* 2002; Zhang *et al.* 2014; Antonio *et al.* 2017; Quadri *et al.* 2018b). This mechanism has been attributed to oxidative stress, caspase-mediated apoptosis, and protein inhibition; specifically, renal phosphatases, dehydrogenases, and sodium transporters have reduced activity at 50-300  $\mu\text{M}$  chronic fluoride serum (Cittanova *et al.* 1996; Partanen 2002; Nabavi *et al.* 2012; Song *et al.* 2014; Song *et al.* 2017; Zhang *et al.* 2014; Antonio *et al.* 2016; Khandare *et al.* 2017; Wahlujo *et al.* 2017). Fluoride damage also causes an imbalance of calcium, magnesium, zinc, iron, and nickel in the kidney

(Suketa *et al.* 1977; Cetin and Yur 2016). Fluoride damage to the kidney can lead to widespread tissue damage; the kidneys maintain blood pH, and impaired kidney function triggers acidosis (Sabatini 1983; Kraut and Madias 2016). Therefore, kidney damage results in greater fluoride uptake as HF in surrounding cells (Sharma *et al.* 2010; Sandhyrani *et al.* 2013).

The liver is primarily responsible for metabolizing metals, detoxifying organo-compounds, and filtering blood from the digestive tract; as such, fluoride toxicity to the liver could greatly alter the body's overall defense systems. Fluoride has been widely reported to cause oxidative stress and lipid damage to the liver (Guo *et al.* 2003; Wang *et al.* 2004; Pereira *et al.* 2016; Lu *et al.* 2017). Namely, fluoride exposure increased lipid peroxidation and saturated fatty acid ratios, and triggered caspase-mediated apoptosis (Vatassery *et al.* 1980; Wang *et al.* 2000; Bouasla *et al.* 2016; Lu *et al.* 2017). Subsequent studies on liver damage over 60 days of fluoride exposure observed damages to the mitochondria, endoplasmic reticulum, and DNA (Campos *et al.* 2017; Pereira *et al.* 2013; 2016; 2018). However, liver damage from fluoride is generally reported in  $\geq 100$  ppm (5 mM) fluoride exposure for over a month in mammals, and over 24 hours in cell culture. Nonetheless, when the liver is damaged from fluoride, the liver releases free radicals and nitric oxide into surrounding cells (Krechniak and Inkielewicz-Stepniak 2005; Zhou *et al.* 2015). Consequently, fluoride-mediated liver damage leads to inflammation and damage to other tissues.

#### Endocrine disruption

Fluoride is believed to be an endocrine disruptor at normal and low iodine levels, although the degree of endocrine disruption differs by fluoride dosage, exposure time, and per individual. Several reports from the mid-1800s to early-1900s observed mammalian populations with high fluoride exposure and thyroid dysfunction (Maumene 1854; McKay 1917; Almond 1923; Stocks 1928; Wilson 1941). While most cases were later attributed to iodine deficiencies in diet, several studies found hypothyroid conditions in patients with normal iodine levels [Stein 1948, Obel 1982, Burgi *et al.* 1984, and Jooste *et al.* 1999]. As a whole, many patients with dental fluorosis also show signs of thyroid dysfunction and lowered hormone synthesis. Fluoride was officially classified by the National Research Council in 2006 as an endocrine disruptor for its ability to inhibit the thyroid at high concentrations in the serum, however, its mechanism of action remains unknown (NRC 2006).

The most widely reported phenotype of endocrine disruption under fluoride is a decrease in the thyroid hormones triiodothyronine (T3) and thyroxine (T4). Studies that have reported a significant decrease of T3 and T4 by fluoride typically involve either patients with dental fluorosis, or mammals exposed to 30-80 ppm (2-4 mM) fluoride for  $\geq 2$  months (Bobek *et al.* 1976; Jiang *et al.* 2015; Jianjie *et al.* 2016). However, a recent study found that fluoride had a mild but detectable impact on T3 concentration in humans exposed to as little as 0.5 ppm (25  $\mu$ M) fluoride (Kheradpisheh *et al.* 2018). The thyroid produces T3 and T4 through the conversion of iodine and tyrosine by the metalloprotein thyroid peroxidase. Thyroid peroxidase activity levels were lowered *in vivo* in patients with fluorosis, which was proposed by the authors to be due to indirect stress effects (Singla and Shashi 2013). Early research suggested that fluoride competes with iodine for import into the thyroid, thus leading to an overall decrease in T3 and T4 production (Chang *et al.* 1934). However, later studies found that fluoride does not accumulate in the thyroid (Wallace 1953; Harris and Hayes 1955; Levi and Silberstein 1955; Galletti and Joyet 1958; Saka *et al.* 1965; Demole 1970). As such, thyroid toxicity is attributed to high fluoride in the blood.

More recently, fluoride has shown to illicit stress signaling and cell damage to the thyroid. Lowered T3 and T4 concentrations cause a rise in thyroid stimulating hormone (TSH) production, which has been shown to occur in patients with fluorosis (Bachinskii *et al.* 1985; Ge *et al.* 2013; Singh *et al.* 2014; Kheradpisheh *et al.* 2018b; Malin *et al.* 2018). Altered TSH can activate adenylate cyclase and stress hormones release into the blood during hypothyroidism (Bech and Madsen 1978). Furthermore, fluoride can directly activate adenylate cyclase and trigger cAMP release (Manganiello and Vaughan 1976; Downs *et al.* 1980; Reese and Hoss 1983; Stadel and Crooke 1988). Hypothyroidism also alters the body's ability to regulate temperature, metabolism, and heart rate, although these phenotypes are also believed to be caused directly by fluoride. On a cellular level, the thyroid undergoes DNA damage, membrane disruption, mitochondrial and endoplasmic reticulum stress, and oxidative stress signaling during fluoride exposure (Sundström 1971; Banji *et al.* 2013; Liu *et al.* 2016; Abdelaleem *et al.* 2018; Yu *et al.* 2018). Cells exposed to both excessive fluoride and iodide showed synergistic toxicity to endoplasmic stress, IRE1 signaling, and DNA damage (Liu *et al.* 2014; 2014b; Jiang *et al.* 2015). Furthermore, several reports show that the oxidative stress and thyroid damage caused by fluoride are partially reduced by addition of antioxidants (Sarkar and Pal 2014; Adedara *et al.* 2016; Yang *et al.* 2016; Abdelaleem *et al.* 2018). As fluorosis leads to higher free radicals, inflammation, and nitric oxide

in the serum, any of these could partially explain thyroid disruption (Susheela and Jethanandani 1994; Reddy *et al.* 2003; Liu *et al.* 2012).

In 2015, the public nominated fluoride for investigation by the United States National Toxicology Program (NTP) for its potential role in non-thyroidal endocrine disruption, cancer, and neurological disorders. After conducting a systematic review, the NTP cited insufficient research on low doses of fluoride to investigate the real risk of these effects (NTP 2017). Research conducted with high dosages of fluoride reports direct and indirect inhibitory effects on the endocrine system, independent of the thyroid. The most notable targets are the pineal gland, adrenal glands, and the parathyroid. The pineal gland lies outside of the blood brain barrier and has the highest calcification rate of any organ in the body (Tan *et al.* 2018). Fluoride has been shown to gradually accumulate in the pineal gland along with calcium (Luke 2001; Kalisinska *et al.* 2014). Few studies have been conducted on whether fluoride accumulation significantly disrupts pineal gland activity. The key study, conducted in 1997, reported that prepubescent gerbils fed 40 ppm (2 mM) NaF daily had lower melatonin production by the pineal gland than the controls, but that melatonin was restored to normal concentrations when the gerbils reached adulthood (Luke 1997). Patients with skeletal fluorosis (the most severe form of fluoride toxicity), are sometimes found to have secondary hyperparathyroidism (Teotia and Teotia 1973). This has been suggested as the body's attempt to restore calcium and phosphate homeostasis, rather than the direct disruption of the parathyroid by fluoride (Faccini 1969; Krishnamachari 1986).

### Neurotoxicity

There has been much debate as to whether fluoride exposure leads to neuronal damage, including Parkinson's disease, Alzheimer's disease, and a reduced IQ. In short, there is not enough evidence to show causation. In order for a molecule to cause neurotoxicity, it must first pass through the blood brain barrier. The blood brain barrier is composed of specialized endothelial cells with greatly reduced membrane permeability (Banks 2009; Chen and Liu 2012). Consequently, most molecules cannot pass from the bloodstream to the brain. The blood brain barrier allows passive diffusion of small, uncharged, lipid soluble compounds (Wong *et al.* 2013). Other compounds, including glucose, amino acids, and nucleosides, are transported with selective channels, pumps, or vesicles. Occasionally another molecule can enter through these channels, such as methylmercury, which complexes with L-cysteine to mimic L-methionine (Aschner

and Aschner 1990; Kerper *et al.* 1992). In the case of fluoride, the ion is highly electronegative and would only pass through the blood brain barrier as HF. As blood has a pH between 7.3-7.45, the majority of fluoride would remain in its unprotonated form (Kellum 2000). Consequently, the levels of fluoride detected in blood tissue are typically much lower than the concentration of fluoride in serum, and generally lower than all other tissues in the body (Carlson *et al.* 1960; Jenkins 1991; Mullenix *et al.* 1995; Whitford 1996; Luke 1989).

Many studies have found correlations between low IQ and high fluoride exposure. Over 50 studies have been conducted in areas with high fluoridated groundwater, and most studies have reported a lower average IQ in the children of those regions compared with children in areas with normal fluoride exposure (Lu *et al.* 2000; Xiang *et al.* 2003; Trivedi *et al.* 2007; Tang *et al.* 2008; Choi *et al.* 2012; Aravind *et al.* 2016). Several other studies have been conducted and found either no correlation between fluoride and IQ, or that high fluoride correlated with higher IQs (Spittle *et al.* 1998; Calderon *et al.* 2000; He *et al.* 2010; Li *et al.* 2010; Broadbent *et al.* 2015). However, these studies do not take into consideration socioeconomic factors, unconscious bias, as well as other possible toxicants in ground water that could be affecting IQ. The areas of interest – primarily China, India, and Mexico – each have groundwater high in other neurotoxicants such as arsenic and mercury (Wang *et al.* 2007; UNEP 2013; Marino and Figoli 2015). In a systematic review of fluoride-IQ studies, the Grandjean lab concluded that if fluoride is a neurotoxicant, it would be over 1,000-times less potent than other known neurotoxicants (Choi *et al.* 2012). Furthermore, any effects on IQ may be indirect due to hypothyroidism symptoms in fluorosis patients. Hypothyroidism is associated with impaired memory and concentration, and fluorosis is many times reported with hypothyroidism (Gottlieb 1999). Because of each of these factors, the correlation of high fluoride exposure and poorer performances in IQ tests does not necessarily imply causation.

In a lab setting, fluoride has caused toxicity to neuronal cells. Neuronal cell lines exposed to  $\geq 60$  ppm (3 mM) NaF showed signs of S-phase cell cycle arrest, DNA damage, oxidative stress, mitochondrial agglutination, and cytoskeleton damage (Bhatnagar *et al.* 2002; Blaylock 2004; Zhang *et al.* 2008; Chen *et al.* 2017; Tu *et al.* 2018). Because the primary function of neurons is synaptic signaling, membrane defects from fluoride exposure reduces the overall activity of neurons. Rats and mice exposed to high fluoride ( $\geq 50$  ppm, or 2.5 mM) showed decreased nicotinic acetylcholine receptor expression, lowered acetyl cholinesterase activity, damaged myelin and microtubules, activation of microglia, and expression of

mitochondrial-mediated apoptotic markers Bax and Bcl-2 in neuronal cells (Vani and Reddy 2000; Long *et al.* 2002; Chen *et al.* 2003; Basha and Sujitha 2012; Lou *et al.* 2014; Niu *et al.* 2015; 2018; Yan *et al.* 2015; Yang *et al.* 2018). As such, high concentrations of fluoride have been suggested to lower the learning ability of these mammals (Niu *et al.* 2009; Han *et al.* 2014). Concentrations of fluoride  $\leq 50$  ppm (2.5 mM) showed no significant brain damage (Varner *et al.* 1998; Shivarajashankara *et al.* 2002). Like many organs under fluoride exposure, brain tissue and neuronal cells show increased in oxidative stress signaling, lipid peroxidation, and free radical production (Flora *et al.* 2009; Kaur *et al.* 2009; Dec *et al.* 2017). Total brain lipid content during 100 ppm (5 mM) fluoride exposure is reduced due to oxidative stress, most notably phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine (Guan *et al.* 1998; Shao *et al.* 2000). Consequently, treatment with antioxidants partially rescues fluoride toxicity to neuronal cells (Chinoy and Shah 2004; Reddy *et al.* 2009; Nallagouni and Reddy 2017).

Several studies have argued that fluoride damages neuronal cells through an indirect mechanism. The Rigalli lab suggested that the alteration of glucose and insulin homeostasis in the blood during fluoride exposure could lead to nutrient depletion and downstream stress in the nervous system (Lombarte *et al.* 2016). However, the Nowak lab reported an enhancement in glucose uptake in brain tissue upon treatment of rats with 50 ppm (2.5 mM) fluoride (Rogalska *et al.* 2017). Others have argued that the release of free radicals by either the intake of  $\geq 60$  ppm (3 mM) NaF, or the intragastric injection of  $\geq 20$  mg/kg body weight NaF – about four times more concentrated than that needed to cause acute gastrointestinal toxicity in humans – could have negative effects on neuronal tissue, particularly the hippocampus (Bhatnagar *et al.* 2002; Pan *et al.* 2015; Shanmugam *et al.* 2018). Again, these studies rely on exposure to high doses of fluoride, well above that typically found in fluoridated water.

High doses of aluminum-fluoride are more likely to enter the blood brain barrier and cause neurotoxicity compared with sodium fluoride. Aluminum fluoride has a different mode of distribution in the body than the fluoride anion, showing no evidence of accumulation in the liver and kidney. Aluminum fluoride (AlF<sub>3</sub> or AlF<sub>4</sub>) are phosphate mimics, and could theoretically cross the blood brain barrier through phosphate transporters (Strunecka *et al.* 2002). Aluminum fluoride exposure in rats exposed to 10 ppm NaF and 100 ppm AlCl<sub>3</sub> in combination for 30 days showed neuronal shrinkage and inhibition of acetylcholinesterase activity (Akinrinade *et al.* 2015). Furthermore, high aluminum fluoride was found to cause more

histopathological changes to brain tissue than sodium fluoride, particularly in the neocortex and hippocampus (Varner *et al.* 1998; NRC 2006). However, it is possible that the neurotoxicity is due to free aluminum. Various studies have found that high fluoride in the serum enhances aluminum uptake in the brain, while high aluminum reduces fluoride uptake (Spencer *et al.* 1980; Ahn *et al.* 1995; Varner *et al.* 1998; Strunecka *et al.* 2002). Furthermore, several studies of brain defects found the highest association with aluminum, not fluoride levels (Forbes *et al.* 1991; Kraus and Forbes 1992; Jacqmin *et al.* 1994). Free aluminum acts as a neurotoxicant, disrupting the cell membrane integrity of the blood brain barrier, activating the innate immune response, and potentially increasing dementia (Banks and Kastin 1989; Armstrong *et al.* 1996; Forbes *et al.* 1995; 1996; Martyn *et al.* 1997; Rondeau *et al.* 2009). Both aluminum and aluminum fluoride can react with oxygen to form aluminum oxide, prevalent in Alzheimer patients (Still 1994; Campbell 2002; Krewski *et al.* 2007). As such, aluminum could be at least partially responsible for fluoride neurotoxicity.

#### Reproductive/Developmental Toxicity

Monitoring of fluoride distribution shows that the ion travels to the testes and uterus, and can be found in both placental and fetal tissue (Gupta *et al.* 1993; Malhotra *et al.* 1993). Pregnant mice treated with 150 ppm (7.5 mM) fluoride resulted in oocytes with damaged mitochondria and cytoskeletons (Liang *et al.* 2015). At 250 ppm (13 mM) fluoride, fertilized oocytes in rats resulted in pups with damaged skeletons (Collins *et al.* 2015). Treatment of non-pregnant rats with  $\geq 100$  ppm (5 mM) fluoride significantly reduces reproductive hormone production, sperm count, follicle maturation, and damages the endometrium (Wan *et al.* 2006; Zhou *et al.* 2013). Similarly, patients with fluorosis have been reported to have lower testosterone and estradiol levels than normal, and potentially a lower fertility rate (Freni 1994; Susheela and Jethanandani 1996; Ortiz-Perez *et al.* 2003; Hao *et al.* 2010; Zhou *et al.* 2016). These effects have been linked to oxidative stress and lipid peroxidation; treatment with antioxidants reduce fluoride toxicity to reproductive organs (Zhang *et al.* 2006; Guney *et al.* 2007; Saumya and Basha 2017). Regardless, these studies were conducted at much higher fluoride concentrations than typically found in nature. Systematic reviews on developmental effects in human populations with fluoridated water found no significant correlation between fluoride exposure and birth defects (ATSDR 1993; 2003).



## Diabetes

Given that fluoride alters cellular metabolism, there have been concerns about the effect of fluoride on sugar homeostasis and diabetes. Prolonged exposure to high fluoride inhibits glycolysis and ATP production. Cells respond to fluoride stress, as well as to many acids, by increasing glucose uptake (Hay and Paul 1967; Rogalska *et al.* 2017). The direct intraperitoneal injection of mammals with high fluoride, resulting in at least 0.1 mg/L total fluoride in the blood, can lead to higher blood glucose (McGown and Suttie 1977; Suketa *et al.* 1985; NRC 2006). However, studies with rats fed 15-50 ppm (0.8-3 mM) fluoride showed either no change, or a decrease in serum glucose levels (Lupo *et al.* 2011; Lobo *et al.* 2015; Malvezzi *et al.* 2019).

Fluoride exposure has also been demonstrated to alter insulin concentrations in the blood. Fluoride reversibly inhibits insulin secretion, leading to an overall reduced insulin concentration in the serum (Rigalli *et al.* 1990; Menoyo *et al.* 2005). However, exposure to low (10 ppm, or 0.5 mM) doses of fluoride enhances insulin sensitivity (Lobo *et al.* 2015). In all, chronic exposure to high fluoride may partially contribute to diabetes, while low fluoride exposure may be protective against diabetes.

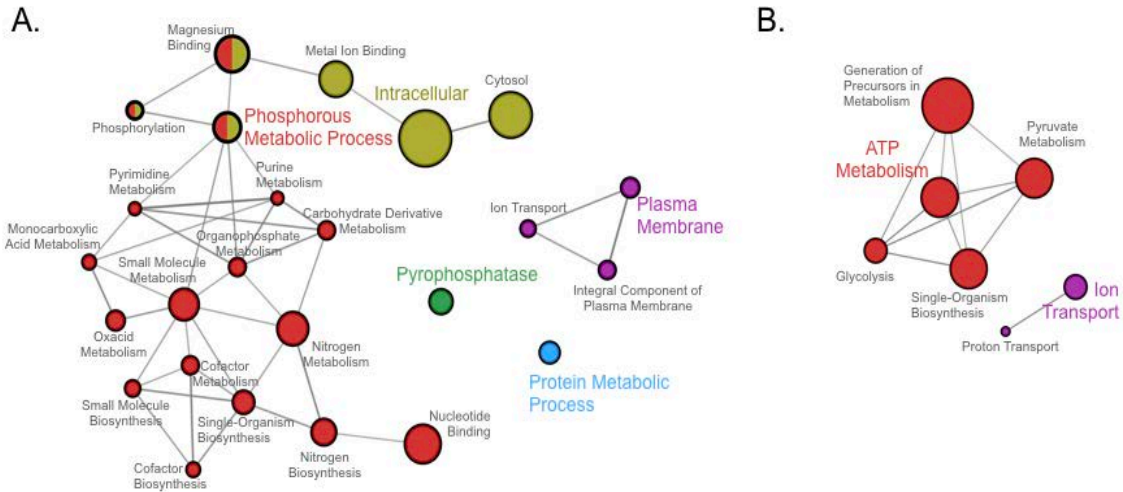
## **VIII. Resistance to fluoride**

Many organisms have evolved defense mechanisms against fluoride. The first discovery of a fluoride-specific defense pathway occurred in 2012, when the Breaker lab identified a region of RNA, known as a riboswitch, conserved in many bacteria and archaea (Baker *et al.* 2012). Riboswitches are located on some mRNA and control downstream gene expression upon binding to ligands. In the case of the fluoride riboswitch, the RNA coordinates with three magnesium ions to bind fluoride at a  $K_D$  of 60  $\mu$ M (1.1 ppm) (Ren *et al.* 2012). Over 2,000 examples of the fluoride riboswitch were identified, and found to control expression of many genes linked to fluoride resistance (Baker *et al.* 2012). Included in this list were genes functioning in oxidative stress, DNA repair, and intracellular acidification. There were also genes for proteins known to be inhibited by fluoride, including enolase,  $\text{Na}^+/\text{H}^+$  antiporters, and pyrophosphatase. Thirdly, there were two newly discovered fluoride channels:  $\text{EriC}^{\text{F}}$  and Fluc.

The transporters  $\text{EriC}^{\text{F}}$  and Fluc confer significant fluoride resistance.  $\text{EriC}^{\text{F}}$  is a member of the Clc family of membrane proteins, and acts as a  $\text{F}^-/\text{H}^+$  antiporter (Lim *et al.* 2013). Fluc is believed to be a channel whose driving force for fluoride efflux is the electrochemical gradient of the bacterial plasma membrane (Stockbridge *et al.* 2013; Ji *et al.* 2014). Fluoride exporters are an essential part in mediating fluoride's toxic

effects, conferring a 200-fold increase in resistance to bacterial growth arrest. In yeast this resistance is even more pronounced, with over 1000-fold increased resistance to fluoride (Li *et al.* 2013). While the fluoride transporter is conserved across many species of eukaryotes and prokaryotes, no homolog has been identified in vertebrates. Nonetheless, fluoride sensitivity varies across species and tissue-type, suggesting there is an as yet undiscovered mechanism of defense.

Microbes have evolved multiple mechanisms of fluoride resistance. These resistance factors are generally found by either isolating organisms from areas with high fluoride, or exposing cells to fluoride in a laboratory. Several studies identified fluoride-resistant bacteria that express higher copies of fluoride transporters, as well as higher copies of known fluoride targets (Liao *et al.* 2015; 2016; Liu *et al.* 2017). In one of the few reports that did not find an altered fluoride channel, fluoride-resistant *S. mutans* adjusted their composition of fatty acids and had enhanced general acid resistance (Zhu *et al.* 2012). This correlation of fluoride-resistance with acid resistance has been widely observed, although the mechanism has never been found (Sheng and Liu 2000; Marquis *et al.* 2003). A DNA microarray of a resistant strain of *A. ferrooxidans* offered one of the most complete pictures of fluoride tolerance, showing a change in expression of genes related to metabolism, protein synthesis, and cell membrane maintenance (Ma *et al.* 2016). As a whole, microbes appear to gain fluoride resistance by increasing expression of fluoride transporters and protein targets of fluoride inhibition, most notably ATPases and glycolytic enzymes (Fig. 4).



**Figure 4: Global network of cellular processes involved in the resistance of bacteria to fluoride. (A)**

Conserved molecular functions and cellular components of (A) genes regulated by the fluoride riboswitch, as reported by Weinberg *et al.* 2010, and (B) altered genes in fluoride resistant bacteria (Zhu *et al.* 2012; Liao *et al.* 2015; 2016; Ma *et al.* 2016; Liu *et al.* 2017). Genes were converted to their *E. coli* homologs, and duplicates were discarded. Data was analyzed on Cytoscape using ClueGO. Node size corresponds with the number of genes per category, and similar colored nodes denote a similar cluster in function.

Mammalian cells are also capable of gaining fluoride resistance. A study comparing mice that were either resistant or sensitive to developing fluorosis showed differences on chromosomes 2 and 11, although these differences were comprised of nearly 2,000 genes (Everett *et al.* 2009). An RNA-Seq study investigating gene expression differences in a fluoride-resistant mouse adipose cell line showed increased expression in genes related to general stress response, protein synthesis, and cell membrane maintenance (Ran *et al.* 2017). Studies on mammalian resistance to fluoride have not yet found a link with overexpressing glycolytic enzymes, although RNA-Seq of rats after 20-60 days exposure to 50 ppm (3 mM) NaF showed an increased expression of genes related to glucose uptake (Pereira *et al.* 2018). Continued investigation into fluoride toxicity and the concurrent mechanism of resistance across species is a much-needed avenue for fully understanding the biological effects of fluoride exposure.

**Summary:**

The issue of whether fluoride is safe depends on the sensitivity of the organism, the concentration of fluoride, and the conditions by which fluoride is administered. Fluoride has both positive and negative effects. Low fluoride levels decrease cavities and partially restores the minerals in teeth. High levels of fluoride lead to protein inhibition, a release of free radicals, disruption of metal homeostasis, and tissue damage. The question, then, becomes how much fluoride an organism will encounter during its lifetime.

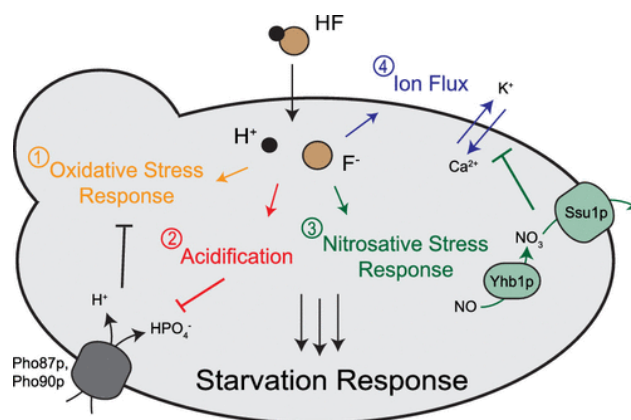
Most organisms are in regions with low to mid-range fluoride, and are at low risk to experience fluoride toxicity. Consequently, the majority of the world's population have no visible signs of fluorosis. However, the inhabitants of certain regions around the world, including in India, China, and Africa, have to be particularly aware of the fluoride levels to which they are exposed. In these areas, there is emerging interest on the safe removal of fluoride from the groundwater and air.

## Chapter 2: Nitrate and phosphate transporters rescue fluoride toxicity in sensitized yeast

This chapter has been published in *ACS Chemical Research in Toxicology*, 32(11):2305-2319 (2019). (<https://doi.org/10.1021/acs.chemrestox.9b00315>). All experiments reported were conducted by myself. The manuscript and figures were prepared by myself and Scott A. Strobel.

### Abstract

Organisms are exposed to fluoride in the air, water, and soil. Yeast and other microbes utilize fluoride channels as a method to prevent intracellular fluoride accumulation and mediate fluoride toxicity. Consequently, deletion of fluoride exporter genes (FEX) in *S. cerevisiae* resulted in over 1000-fold increased fluoride sensitivity. We used this FEX knockout strain to identify genes, that when overexpressed, are able to partially relieve the toxicity of fluoride exposure. Overexpression of five genes, SSU1, YHB1, IPP1, PHO87, and PHO90, increase fluoride tolerance by 2- to 10-fold. Overexpression of these genes did not provide improved fluoride resistance in wild-type yeast, suggesting that the mechanism is specific to low fluoride toxicity in yeast. Ssu1p and Yhb1p both function in nitrosative stress response, which is induced upon fluoride exposure along with metal influx. Ipp1p, Pho87p, and Pho90p increase intracellular orthophosphate. Consistent with this observation, fluoride toxicity is also partially mitigated by the addition of high levels of phosphate to the growth media. Fluoride inhibits phosphate import upon stress induction and causes nutrient starvation and organelle disruption, as supported by gene induction monitored through RNA-Seq. The combination of observations suggests that transmembrane nutrient transporters are among the most sensitized proteins during fluoride-instigated stress.



Graphical Abstract

## Introduction.

Fluoride is the 13<sup>th</sup> most abundant element in the Earth's crust (Smith *et al.* 1977). While fluoride is pervasive throughout the environment, it is most highly concentrated in areas with industrial fumes, volcanic activity, or marine sedimentation (Jha *et al.* 2011; Vithanage and Bhattacharya 2015). Consequently, the exposure of organisms to fluoride varies with their geographical location. Seawater averages at 1.2–1.5 ppm (60–80  $\mu\text{M}$ ), while soil ranges from 100 to 620 ppm (Jha *et al.* 2011). Many countries, including the U.S., add fluoride to drinking water at 0.7–1.5 ppm (40–80  $\mu\text{M}$ ) as a method of improving dental hygiene (US DHHS 2015). The Center for Disease Control identified water fluoridation as one of the 10 greatest public health achievements of the twentieth century (CDC 1999).

Exposure to high levels of fluoride over a period of weeks to months produces a myriad of stress effects in organisms. High fluoride causes inflammation, lipid peroxidation, and perturbation of the MAPK and NF- $\kappa\text{B}$  pathways in multicellular model systems (Hivarajashankara and Shivashankara 2003; Shanthakumari *et al.* 2004; Refsnes *et al.* 2014; Luo *et al.* 2017; Chen *et al.* 2019). At a single-cell level, fluoride activates S-phase cell-cycle arrest and metabolic arrest, and damages both the mitochondria and endoplasmic reticulum (Feig *et al.* 1971; Anuradha *et al.* 2001; Kubota *et al.* 2005; Zhang *et al.* 2008; Sharma *et al.* 2008; Fina *et al.* 2014). These effects are linked to the activation by fluoride of oxidative stress and the subsequent generation of free radicals, which causes DNA damage and intracellular acidification (Hamilton 1990; Belli *et al.* 1995; Chen *et al.* 2003; He and Chen 2006; Gassowska *et al.* 2013; Zhou *et al.* 2015). As the predominant effects of fluoride toxicity are inflammation and free radical damage, it is challenging to distinguish between the specific mechanism of fluoride toxicity and its downstream stress effects.

The mechanism of fluoride toxicity is linked to its size and high affinity for metals. Fluoride is approximately the same size and charge as hydroxide, but much more electronegative (3.98 versus 1.34 on the Pauling scale). Consequently, fluoride can displace hydroxide from its coordinating partners, especially metals. In enamel, fluoride displaces the hydroxide in hydroxyapatite to form fluorapatite. Fluorapatite is resistant to demineralization from bacteria, and therefore prevents cavities (ten Cate and Featherstone 1991; Featherstone 1999). On a molecular level, fluoride has been found to directly modify amino acids, such as Arg-48 of cytochrome *c* peroxidase, as well as to displace the hydroxides of Ser/Thr phosphatases, which prevents dephosphorylation activity (Edwards *et al.* 1984; Edwards and Poulos 1990; Schenk *et al.*

2008). More commonly, fluoride binds to metals within metalloprotein active sites, thus inactivating the metalloproteins (Baykov *et al.* 1979; Adamek *et al.* 2005; Qin *et al.* 2006). Fluoride–metal complexes can also be a competitive inhibitor of phosphate in phosphoryl transfer enzymes (Wang *et al.* 1995; Wang *et al.* 2001). Because of its high affinity to metals, fluoride has been shown to inhibit hundreds of proteins at high (millimolar) concentrations *in vitro* (Adamek *et al.* 2005; PDB 2000).

Microorganisms, including bacteria and fungi, have evolved systems to defend themselves against fluoride toxicity. Bacteria utilize riboswitches to activate the transcription of many genes in response to fluoride, including the fluoride exporter Fluc (Baker *e al.* 2012; Stockbridge *et al.* 2013). Fluc is a four-helix transmembrane protein that forms a homodimer (Rapp *et al.* 2006; Stockbridge *et al.* 2014; Last *et al.* 2016). It acts as a highly selective channel to prevent the intracellular accumulation of fluoride (Baker *et al.* 2012; Ji *et al.* 2014). Fluc activity is driven by the electrochemical gradient of fluoride across the cell membrane that would otherwise result in the buildup of fluoride in the cytoplasm (Ji *et al.* 2014). Bacteria lacking Fluc are 200-fold more fluoride sensitive than wild-type (Baker *et al.* 2012). Even stronger effects are seen in the eukaryote model organism *S. cerevisiae*. The constitutively expressed FEX1 and FEX2 (the yeast homologues of Fluc), confer over 1000-fold resistance to fluoride (Li *et al.* 2013). In the absence of a fluoride channel, yeast cannot grow at the concentration of fluoride found in municipal tap water.

The fluoride hypersensitivity of yeast with deletions of both FEX1 and FEX2 (FEX double knockout, or DKO) provides a useful system to investigate fluoride toxicity at low extracellular fluoride. FEX DKO yeast are incapable of removing intracellular fluoride. Consequently, these cells demonstrate signs of toxicity within just a few hours, and at an  $IC_{50}$  of 50  $\mu$ M NaF, as opposed to 75 mM in wild-type (Li *et al.* 2013). The concentration of fluoride needed to cause cell cycle arrest in FEX DKO cells is well below the concentration of fluoride required to inhibit metalloproteins *in vitro* (Adamek *et al.* 2005). This observation, combined with the power of yeast genetic manipulation, offers a valuable opportunity to understand intracellular fluoride toxicity in eukaryotes.

An overexpression screen for genes that confer partial fluoride resistance is a well-documented and high-throughput approach to identify the molecular targets of toxicants and associated pathways (Smith *et al.* 2010; Palmer and Kishony 2014). The screen is composed of a tiled DNA-fragment library inserted onto high copy plasmids. If the DNA fragment encodes a protein target of the toxicant or a protein that mediates

toxicity, then increased expression of that protein confers partial resistance to the toxicant. Overexpression screens have been used to elucidate the mechanism of toxicity for many agents, including methylmercury, tunicamycin, methotrexate, and glyphosate (Rine *et al.* 1983; Naganuma *et al.* 2002; Gaines *et al.* 2010; Arnoldo *et al.* 2014).

Transcriptome sequencing (RNA-Seq) serves as a powerful tool for elucidating the response of a cell to a particular stressor. RNA-Seq monitors global RNA expression by reporting the quantity and types of RNA in cells at a given time (Wang *et al.* 2009). Consequently, any stress response triggered in cells to toxicants, such as fluoride, can be identified using differential expression analysis. RNA-Seq functions as an unbiased approach for monitoring gene induction and repression. This technique has been used to monitor cellular response to a wide variety of stressors, including nanoparticles, viral infections, and environmental pollutants (Sessions *et al.* 2013; Simon *et al.* 2013; Xu *et al.* 2013; Aufauvre *et al.* 2014).

Here we report the results of a gene overexpression screen for fluoride resistance and RNA-Seq to analyze stress pathways during fluoride exposure. We observed patterns consistent with fluoride-induced oxidative stress, nutrient starvation, nitrosative stress, membrane disruption, and metal imbalance. This report furthers our knowledge of fluoride toxicity at the low concentrations that most organisms are exposed to in nature.

### **Experimental Procedures.**

Unless otherwise noted, all experiments were performed in triplicate.

Strains and media: The wild type yeast used in this study was BY4741, and the FEX DKO strain was SSY3, generated using the hphMX4 and kanMX6 resistance cassettes as reported previously (*MATa his3Δ1 leu2Δ0 ura3Δ0 FEX1Δ::kanMX6 FEX2Δ::hphMX*).<sup>40</sup> Sodium fluoride was purchased from Aldrich Chemistry. YPD media was prepared using 2 grams yeast peptone (Becton, Dickinson and Co.), 1 gram yeast extract (Becton, Dickinson and Co.), 100 μL of 1% adenine (Sigma), and 5 mL of 40% glucose (Sigma) per 100 mL total volume in water. YPD agar was prepared using the aforementioned ingredients plus an additional 2 grams of agar (Becton, Dickinson and Co.). Phosphate concentration of YPD media was verified using a commercially available malachite kit (Sigma) against a standard of increasing phosphate. Yeast media with specific phosphate concentrations were prepared using SD buffer without ammonium or phosphate (Formedium), which was supplemented with ammonium, amino acids, glucose, and adenine, as well as



sodium phosphate at the indicated concentrations per experiment (J.T. Baker). Unless otherwise noted, all media were pH adjusted to 6.5-6.54 or as indicated using NaOH (J.T. Baker) and HCl (Macron Fine Chemicals).

Tiling fragments for the overexpression screen are commercially available (Minimal 1588 Plasmid Collection, Open Biosystems/Dharmacon). The fragments were ligated onto pGP564 vectors, although individual genes from tiling hits were testing using pRS426 and pRS426GPD vectors. Plasmids were transformed into yeast using the standard lithium acetate method. pGP564-containing cells were grown and maintained in SD-Leu media/agar, and pRS426-containing cells were grown and maintained in SD-Ura media/agar.

Measurement of intracellular fluoride: Cells at O.D. 10 were added to 2 mL liquid containing YPD +/- NaF and placed in a shaker at 30°C. At 2, 4, 6, 12, 18, and 24 hours, cells were collected, counted, and harvested. The supernatant was removed via centrifugation and collected for analysis. Cells were washed twice with water before being resuspended in 1 mL lysis buffer, containing 1% Triton X-100 (Sigma), 0.1% SDS (American Bioanalytical), and PBS (American Bioanalytical). Cells were then placed on a shaker overnight at room temperature, and in the following morning sonicated, then measured for fluoride content using a fluoride electrode (Cole-Parmer). The electrode was calibrated used a standard curve of fluoride in lysis buffer. Intracellular fluoride concentration was calculated based on cell count and the corresponding dilution factor.

Measurement of cell viability (Flow Cytometry): Flow cytometry was performed as outlined in Shen *et al.*, 2014. Briefly, yeast were made into spheroplasts by incubating in buffer (1.2 M sorbitol, 0.5 mM MgCl<sub>2</sub>, and 35 mM potassium phosphate, pH 6.8) with 1 µL zymolyase (Zymo Research, stored in 500 µL of supplied buffer) at 30°C for 1 hour. Yeast were then resuspended in 500 µL binding buffer (10 mM Hepes/NaOH, 140 mM NaCl, and 2.5 mM CaCl<sub>2</sub>, pH 7.4) with 0.5 µL annexin V (Biolegend) and 0.5 µL propidium iodide (MP), placed on ice, and immediately analyzed with flow cytometry using BD FACS-Aria for cell sorting and BD FACS-Verse for analysis.

Measurement of intracellular ATP (Cell Titer Glo): Intracellular ATP was quantified in yeast starting at O.D. 0.1 using the standard CellTiter-Glo Luminescent Cell Viability kit (Promega), and the concentration was established by comparing samples to a standard curve of increasing dATP.

Measurement of mitochondrial integrity (FUN-1 dye): FEX DKO yeast were suspended in 3 mL YPD +/- 50  $\mu$ M NaF at O.D. 0.1 and placed in a 30°C incubator with shaking. At 4 hours, cells at O.D. 0.1 were resuspended in 1 mL PBS with 1  $\mu$ L FUN-1 and incubated at 30°C water bath for 25 minutes, and then washed twice in PBS. Cells were resuspended in PBS and loaded onto a slide for imaging using a Nikon Eclipse Ti Microscope. Photos of yeast with FUN-1 staining were collected using NIS Elements Software, in which the total fluorescence in the GFP (green) channel was compared with the fluorescence in the TRITC (red) channel.

Measurement of cytoplasmic calcium (Indo-1): Yeast were grown in 3 mL YPD +/- 50  $\mu$ M NaF at starting O.D. 0.1 in a 30°C incubator with shaking. At 2, 3, 6, 12, 18, and 24 hours, cells were collected and counted. Cells at O.D. 0.1 were resuspended into 500  $\mu$ L buffer at pH 5 containing 10  $\mu$ M Indo-1 AM (Abcam), 10 mM dimethyl glutaric acid, 50 mM KCl, and 100 mM glucose. Cells were placed in a 30°C water bath for 1.5 hours. Cells were washed three times in PBS, then resuspended in 100  $\mu$ L PBS. Indo-1 fluorescence was recorded at 450 nm emission and 355 nm excitation using a plate reader.

RNA harvesting for RT-qPCR and RNA-Seq: Yeast were grown in 25 mL YPD at O.D. 0.1. At the indicated time points, cells were spun down twice and resuspended in 1 mL sterile water. RNA was isolated using the protocol by Ares 2012. Afterwards, 10  $\mu$ g of RNA was treated for 20 minutes at 37°C with 1x DNase buffer and 1  $\mu$ L Turbo DNase (Invitrogen) before extraction using phenol:chloroform and ethanol. RNA was resuspended in sterile water. For RNA-Seq, samples were sent to the Yale Center for Genome Analysis for quality analysis with a Bioanalyzer and subsequent poly-A sequencing. RT-qPCR was performed using the Luna® Universal One-Step RT-qPCR Kit (NEB #E3005), using the recommended protocol. Background DNA content of each sample was assessed using primers against an intron segment of actin, and relative RNA across samples were compared using primers against an exon segment of actin.

Serial dilutions on YPD-agar: Yeast were inoculated overnight in 2 mL YPD. The next day, cells were spun down twice and resuspended in 1 mL water at O.D. 1 (denoted as  $10^7$  in figures). 1:10 dilutions were conducted in separate tubes of 1 mL total water to generate  $10^6$ ,  $10^5$ ,  $10^4$ ,  $10^3$ , and  $10^2$  of cells/mL. Of these aliquots, 8.75  $\mu$ L were added sequentially to YPD-agar plates in increasing fluoride. The plates were placed on the counter at room temperature until no liquid was visible, then turned upside-down and placed in a 30°C incubator for two days.

Assessing effects of compounds on growth (liquid growth assay): Liquid growth assays were conducted as outlined in Li *et al.*, 2013. Yeast were added to a 24-well plate at O.D. 0.1. Each well contained 1 mL liquid media of increasing toxin (usually fluoride), as specified per experiment. The plate was shaken at medium speed in a plate reader at 30°C for a period of 24 hours, with the absorbance at 600 nm measured every 3 minutes. The data were plotted on Prism, and IC<sub>50</sub> values were calculated by comparing the fold-change in growth by area under the curve (AUC) compared to growth of cells in YPD alone. Unless otherwise noted, media was altered to pH 6.5-6.54 using NaOH and HCl.

Measuring efflux of nitrosative stress substrates (nitric oxide, nitrate, nitrite, and sulfite): Each substrate was measured using the recommended company protocols. Nitrate and nitrite were assessed using a colorimetric kit (Caymen Chemicals), sulfite using an enzymatic kit (Megazyme), and nitric oxide using DAF-FM diacetate (Caymen Chemicals).

Inductively coupled plasma mass spectrometry (ICP-MS): Yeast were grown at starting O.D. 10 in 3 mL YPD +/- 50 µM NaF at 30°C for 24 hours. Afterwards, cells were washed three times in water. Yeast at O.D. 20 were suspended in 1 mL of 20% nitric acid. The tubes were sealed and placed in a 95°C heating block overnight. In the morning, tubes were placed on ice to cool. For each sample, three tubes of 1 mL each were combined (3 mL total), and diluted in water to 2% nitric acid. The three tubes combined represented n=1, and this was repeated for three days (n=3). Samples were analyzed using a Perkin Elmer ICP-MS Elan DRC-e. The cell lysate was assessed for concentrations of potassium, phosphorous, oxidized phosphorous, oxidized sulfur, silicon, copper, magnesium, iron, zinc and calcium, then quantified using a standard curve.

Intracellular phosphorous quantification (<sup>31</sup>P NMR): Nuclear magnetic resonance (NMR) was conducted based on the requirements outlined by Campbell-Burk and Shulman, 1987. Yeast were grown 12 hours, then resuspended at 25% weight/volume in 300 µL water, 150 µL D<sub>2</sub>O, 100 µL YPD, and 0.75 µL 0.5 M MDP (methylene diphosphonate, or medronic acid). After resuspension, 550 µL was transferred to an NMR tube for immediate analysis. NMR was conducted on a Bruker 400 MHz Broadband Probe with 514 scans taken at 1-second intervals.

Monitoring rate of phosphate import (<sup>32</sup>PO<sub>4</sub> influx): The assay was conducted as in Rothstein and Donovan, 1963, and Wykoff and O'Shea, 2001. Briefly, cells were grown for three hours at a starting O.D. 0.67 in 5 mL YPD +/- fluoride. O.D. was again measured, and cells were washed three times in SD (synthetic

defined, or standard minimal) media lacking phosphate. To cells at O.D. 1, SD media was added containing 10 mM PO<sub>4</sub> with <sup>32</sup>PO<sub>4</sub> tracer +/- fluoride as specified. Cells were then placed on a shaker for the indicated time points. Cells were spun three times and washed with 500 mM PO<sub>4</sub> to outcompete import of <sup>32</sup>PO<sub>4</sub>, and the initial supernatant and final pellet were collected and analyzed for radioactivity. All buffers were at pH 6.5 except for the experiments testing pH dependence. In these cases, cells were incubated in increasing concentrations of acetic acid for 30 minutes until they reached the desired pH<sub>intra</sub> (verified using 5(6)-CFDA dye under its standard protocol), then the cells were quickly washed three times and resuspended in buffer at pH 6.5. For experiments with buffer below pH 6.5, cells were grown for three hours in pH 6.5 buffer, then transferred to buffer acidified by HCl at the same time as <sup>32</sup>PO<sub>4</sub> exposure. Results were reported as in the Rothstein lab, setting 100% as uptake in control cells at 60 minutes, and comparing phosphate uptake per cell over time.

RNA-Seq analysis: HiSat2 was used for alignment, HTSeq for generating count data, and DESeq2 for differential expression analysis. The yeast genome from Ensemble was used as a reference. Gene annotation from yeastgenome.org was used for assembling the functional classification chart, and results were plotted using Prism software.

Measurement of plasma membrane electrochemical potential (diS-C3): Yeast were grown at starting O.D. 0.1 in 2 mL YPD +/- 50 μM NaF at 30°C for 3 hours. Yeast were then washed twice with water, and transferred to 24-well plates at O.D. 0.4 in 1 mL of 10 mM citrate phosphate, pH 6.0 with 2% glucose. 0.5 μL of diS-C3 (3,3'-dipropylthiadicarbocyanine iodide) was added to each well, and the fluorescence emission spectra was measured every 6 minutes on a spectrofluorometer at λ<sub>excitation</sub> = 514, and λ<sub>emission</sub> over 543 to 690 nm at a two-step interval. After 30 minutes, either 10 μM FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone), 30 mM HCl, or 50 μM NaF were added to the denoted wells, and reading immediately resumed up to 70 minutes. The reported relative fluorescence units are each the max fluorescence emission, which was λ<sub>573</sub>.

## **Results.**

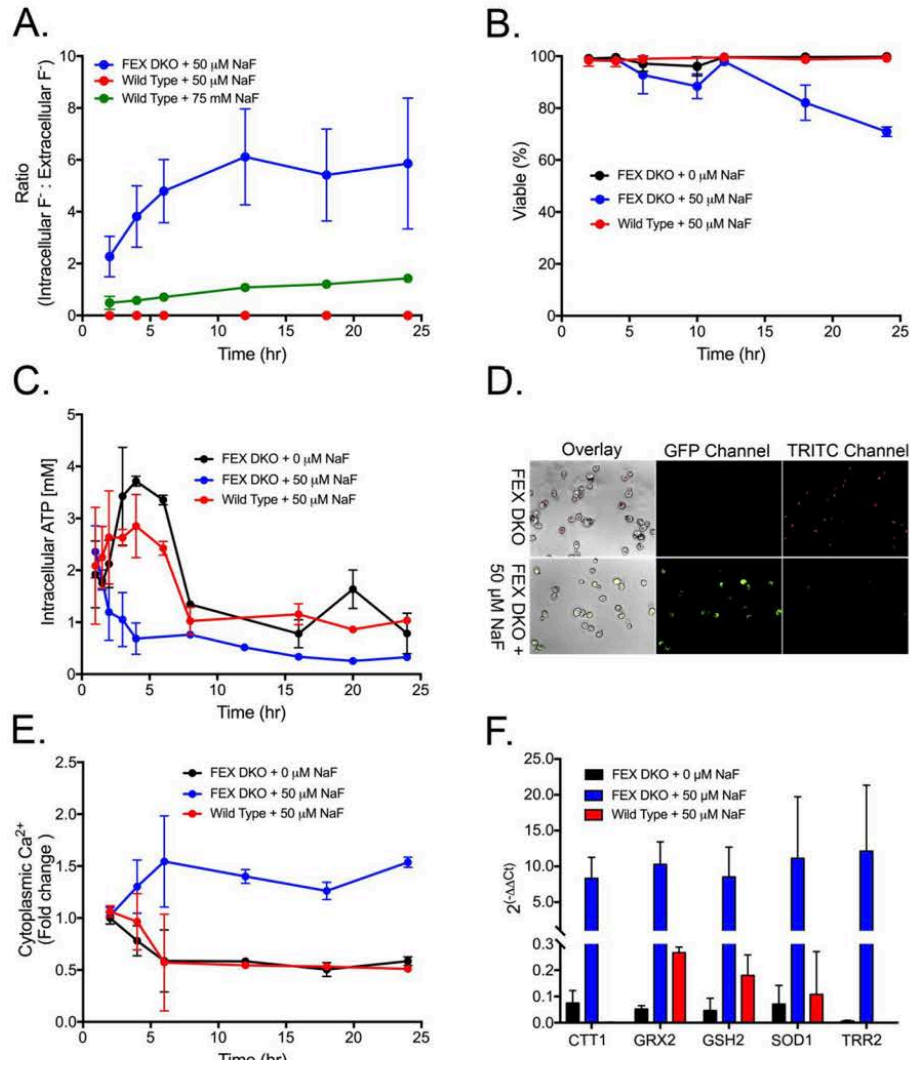
***FEX double-knockout cells are hypersensitive to fluoride exposure*** - Yeast lacking functional fluoride transporters accumulate intracellular fluoride, resulting in hypersensitivity. We monitored intracellular fluoride at the IC<sub>50</sub> of the wildtype and FEX DKO yeast strains. Wildtype yeast exposed to fluoride at its IC<sub>50</sub>

for 24-hour growth (75 mM NaF) equilibrated external and internal fluoride concentrations at approximately a 1:1 ratio (Fig. 1A).

This is consistent with the previous proposal that FEX acts as a channel rather than a pump, and its activity is driven by the fluoride gradient across the membrane (Ji *et al.* 2014). Intracellular fluoride in wildtype at the  $IC_{50}$  for FEX DKO (50  $\mu$ M NaF) was below the detection limit of the electrode. Conversely, FEX DKO yeast exposed to 50  $\mu$ M NaF accumulated fluoride up to an intracellular concentration of 300  $\mu$ M within 24 hours (Fig. 1A). This intracellular concentration, while at a higher ratio of inside fluoride to outside fluoride compared with wildtype, is much lower than the total intracellular fluoride concentration of wildtype at their respective  $IC_{50}$ 's. This suggests that FEX DKO yeast both accumulates fluoride, and is more sensitive to intracellular fluoride. As the only known role for FEX is to remove fluoride, the enhanced sensitivity to fluoride in the absence of FEX is mostly likely due to increased intracellular fluoride toxicity.

We hypothesized that removal of FEX would result in a fast onset in hallmarks of fluoride toxicity. Typical markers of fluoride exposure include growth arrest, loss of metabolic activity, and oxidative stress (Barbier *et al.* 2010). While a few toxicity studies have been conducted in yeast, the vast majority of fluoride studies were conducted in mammalian cell cultures. Mammalian tissue typically take days, or even weeks, to show toxicity phenotypes when exposed to millimolar fluoride (Walton and Eisenmann 1974; Deng *et al.* 2017; Pereira *et al.* 2018). In contrast to these observations, the FEX DKO yeast display arrested growth at an  $IC_{50}$  of just 50  $\mu$ M NaF and begin losing viability after just 12 hours of exposure (Fig. 1B).

We tested the onset of stress effects from fluoride in the hypersensitive FEX DKO yeast. Specifically, we investigated the hallmark phenotypes of metabolic arrest and oxidative stress. Intracellular ATP concentration, which provides an assessment of total metabolic activity and cell viability, was measured using a luciferase-based assay. Fluoride decreased intracellular ATP (Fig. 1C). Cells exposed to fluoride did not undergo the three-fold increase in ATP concentration that was observed in untreated cells as they entered log phase. Furthermore, fluoride-treated cells maintained two-to-three-fold less intracellular ATP over 24 hours compared to untreated cells.



**Figure 1: Fluoride toxicity in the fluoride sensitive FEX double knockout yeast.** (A) Ratio of intracellular fluoride to extracellular fluoride measured by a fluoride electrode. (B) Cell viability over time, as measured by flow cytometry. Viability was determined as the percent of cells in the lower left gate after staining with propidium iodide and annexin V. (C) Measurement of intracellular ATP using cell titer glo and (D) mitochondrial activity as the incorporation of FUN-1 dye at 6 h. (E) Fold change in the concentration of cytoplasmic calcium over time, assed using Indo-1. (F) RTqPCR of oxidative stress response genes at 6 h of growth. The change is compared with gene expression at 1-h growth in YPD without fluoride and using actin for normalization.

Loss of ATP can occur from either glycolytic arrest, or respiratory arrest. We monitored mitochondrial activity using FUN-1 dye, and observed significant reduction of mitochondrial activity after 6 hours of fluoride exposure, as indicated by a shift from red to green fluorescence (Fig. 1D). Decreased mitochondrial activity is generally linked to oxidative stress (Ott *et al.* 2007). Cytoplasmic calcium, an early step in oxidative stress signaling, increased within 2 to 4 hours, and peaked at 6 hours of growth (Fig. 1E). As expected, subsequent RT-qPCR indicated increased expression of oxidative stress genes at 6 hours (Fig. 1F). Overall, these data suggest that ATP production is depleted prior to the loss of mitochondrial activity and induction of oxidative stress.

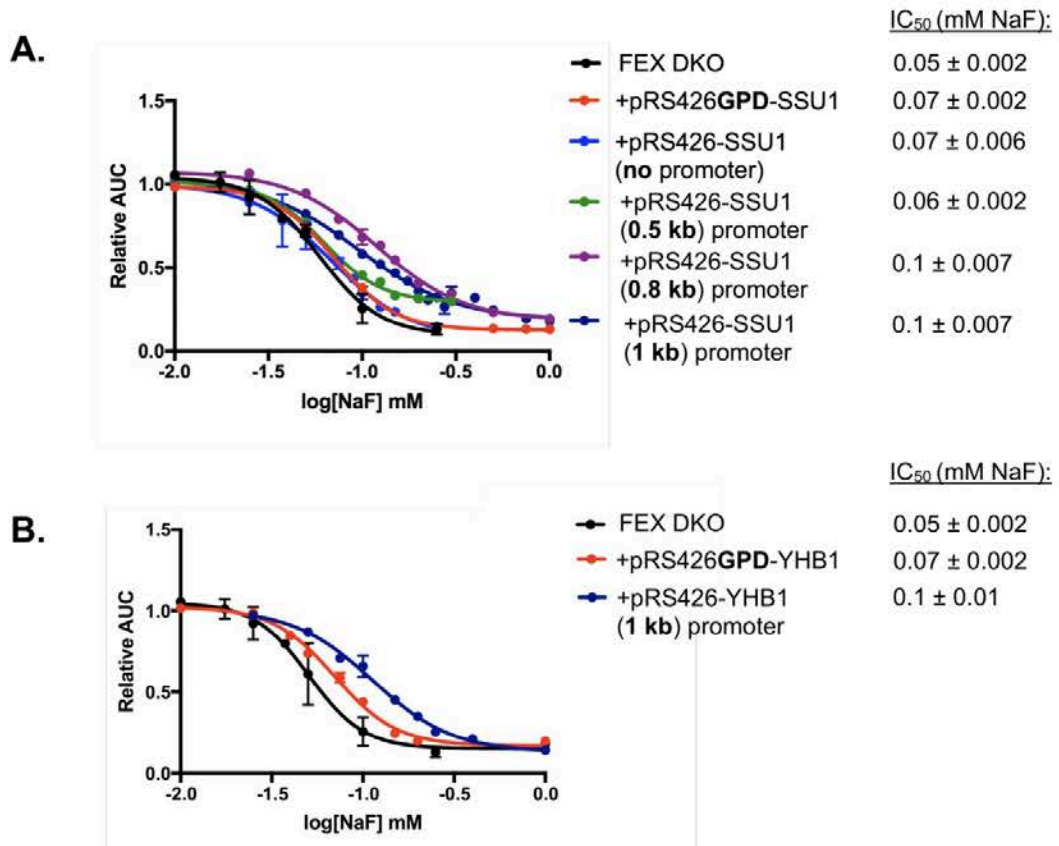
***Overexpression of genes involved in nitrosative stress response and orthophosphate accumulation confer fluoride resistance*** - Fluoride could potentially inhibit hundreds of proteins and activate a myriad of stress pathways. A major challenge in the study of fluoride toxicity is to identify which pathways are directly influenced by fluoride. An overexpression screen using a tiled array provides a high-throughput method to identify important genes that mediate fluoride toxicity. Overexpression screens are composed of DNA-fragment libraries, which are expressed in higher copy numbers within a cell. DNA fragments containing a protein relevant to a mediating toxicity from a particular stressor will confer partial resistant to that stressor when expressed in higher copy numbers.

A commercially-available DNA tiling library was expressed on high-copy plasmids, pooled together, and transformed into FEX DKO yeast. The yeast were screened for their ability to grow on progressively higher concentrations of fluoride. One hundred yeast colonies able to grow in 250  $\mu$ M NaF were selected, and the plasmids isolated and sequenced. Approximately half of the colonies contained either of the two FEX genes. These were not pursued further. The remaining fluoride resistant colonies contained one of five distinct genome fragments, each with 6-8 genes encoded within the fragments.

To identify which gene was responsible for conferring fluoride resistance within each of the five fragments, we generated high copy plasmids containing just one annotated gene from each fragment. These were expressed under the control of the constitutive promoter GPD. Three genes that conferred resistance were identified using this approach: PHO87, PHO90, and IPP1. All three of these genes have well-established functions. Each corresponding protein increases the total intracellular orthophosphate concentration. Pho87p and Pho90p are both constitutively expressed transmembrane proteins that act to import orthophosphate

Wykoff and O’Shea 2001; Bun-Ya *et al.* 1996; Ghillebert *et al.* 2011). Ipp1p is a cytoplasmic inorganic pyrophosphatase that catalyzes the conversion of pyrophosphate into two orthophosphates (Kornberg 1962; Kolakowski *et al.* 1988).

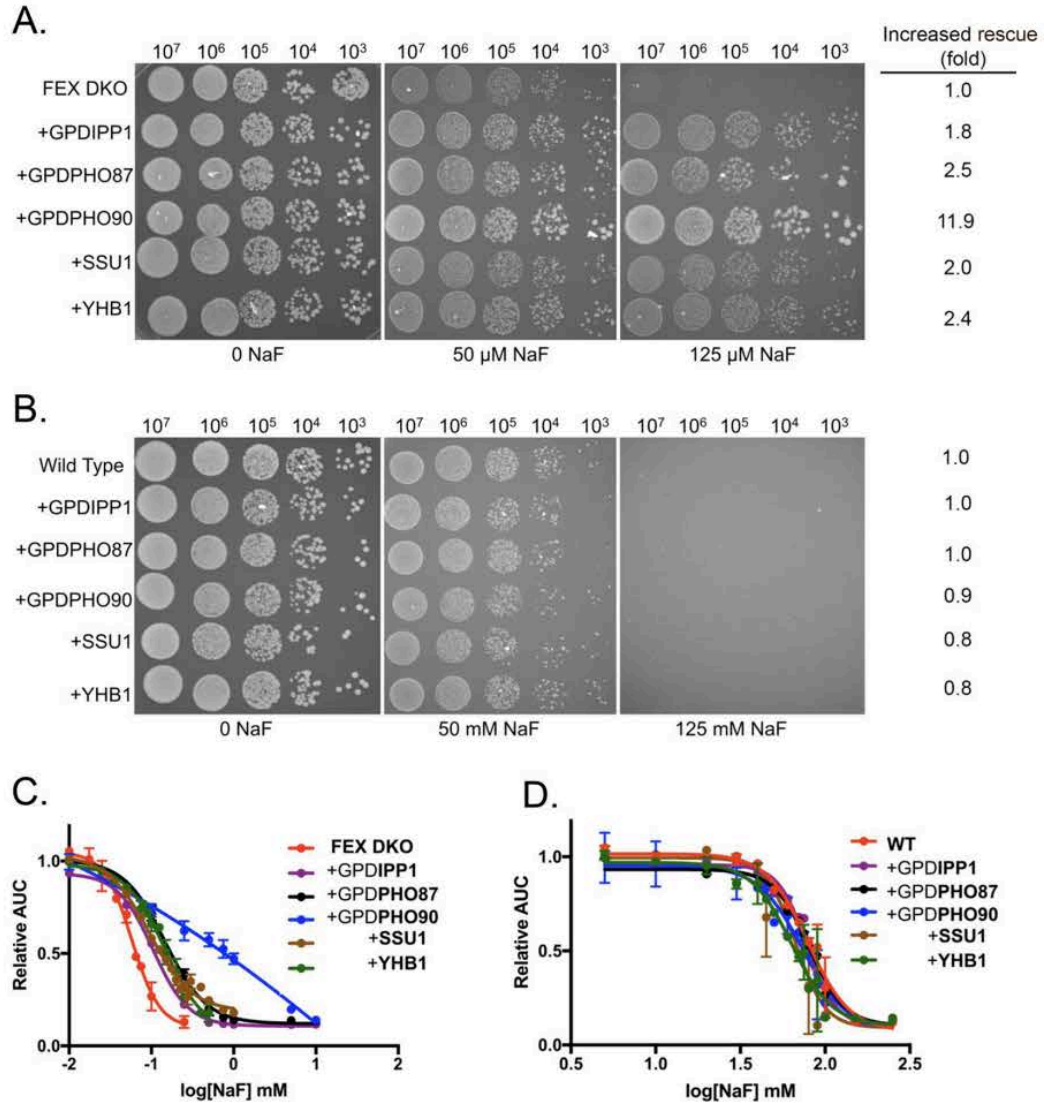
Two other gene fragments conferred resistance to fluoride, but expression of individual genes within that fragment with a GPD promoter did not confer resistance. We sequentially increased the segment genome fragment onto a plasmid without a GPD promoter, and found that two genes, SSU1 and YHB1, also conferred partial fluoride resistance, but only when controlled by their native promoters (Fig. 2). YHB1 required 1 kilobase of its upstream promoter sequence, while SSU1 required 0.8 kilobase of its promoter. Yhb1p is a flavohemoprotein that acts as a nitric oxide oxidoreductase (Cassanova *et al.* 2005). Ssu1p is a transmembrane exporter of both sulfite and nitrate (Avram and Bakalinsky 1997; Park and Bakalinsky 2000; Cabrera *et al.* 2014).



**Figure 2: Effect of promoter for SSU1 and YHB1 on fluoride toxicity in FEX DKO *S. cerevisiae*.** Liquid growth assay over 24 hours of FEX DKO yeast containing pRS426 plasmids with varying promoter lengths preceding (A) SSU1 or (B) YHB1.

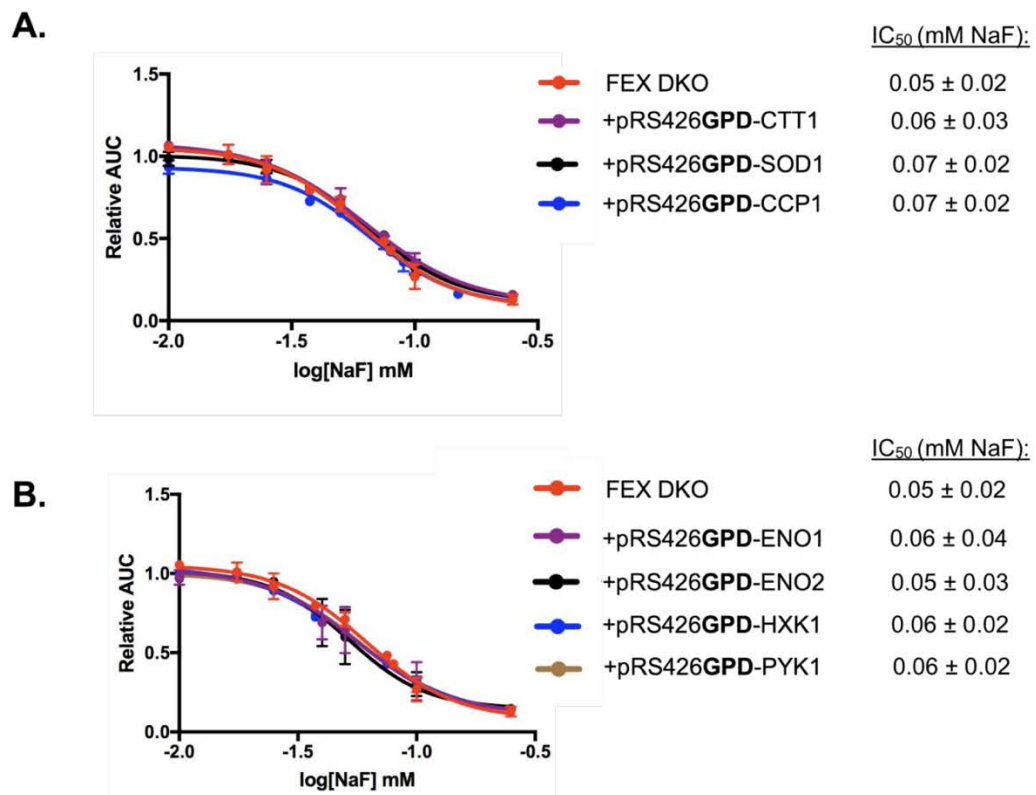


We next tested the extent of fluoride resistance that resulted from the overexpression of individual genes. Yeast grown in liquid culture over 24 hours had two-fold greater fluoride resistance with high-copy plasmids containing IPP1, PHO87, SSU1, or YHB1, and ten-fold greater resistance from the plasmid containing PHO90 (Fig. 2C). With the exception of IPP1, which is a known target for fluoride inhibition, none of these genes have previously been linked to fluoride. Given that the wild type and FEX DKO yeast have substantially different sensitivities to fluoride, we tested if the genes that confer resistance in FEX DKO also conferred improved fluoride resistance in the wild type background. Wild type yeast with high-copy plasmids expressing either IPP1, PHO87, PHO90, SSU1, or YHB1 showed no observable increase in fluoride resistance (Fig. 3D). The mechanism by which these genes confer partial fluoride resistance therefore appears to be specific to low fluoride concentrations.



**Figure 3: Overexpression of proteins conferring fluoride resistance.** Serial dilutions of (A) FEX DKO or (B) wild-type cells  $\pm$  pRS426 plasmids, tested for growth on YPD-agar supplemented with the designated concentrations of fluoride. On the right of the serial dilutions is the fold-change in fluoride resistance based on the change in IC<sub>50</sub>'s. Liquid growth assay of (C) FEX DKO and (D) wild-type cells  $\pm$  pRS426 plasmids over a 24-h period in increasing fluoride. SSU1 and YHB1 plasmids also contain 1 kb of the corresponding gene's native promoter.

Fluoride is well known to cause oxidative stress and metabolic arrest. Strikingly, no glycolytic enzymes or oxidative stress response proteins were identified in the overexpression screen. As a control, we overexpressed seven genes that were previously reported as *in vitro* targets of fluoride, including three genes in the glycolytic pathway, and four linked to oxidative stress (Adamek *et al.* 2005). These genes - ENO1, ENO2, HXK1, SOD1, CTT1, CCP1, and PYK1 - when individually cloned into a high copy plasmid and transformed into FEX DKO, yielded no significant resistance to fluoride toxicity (Fig. 4).



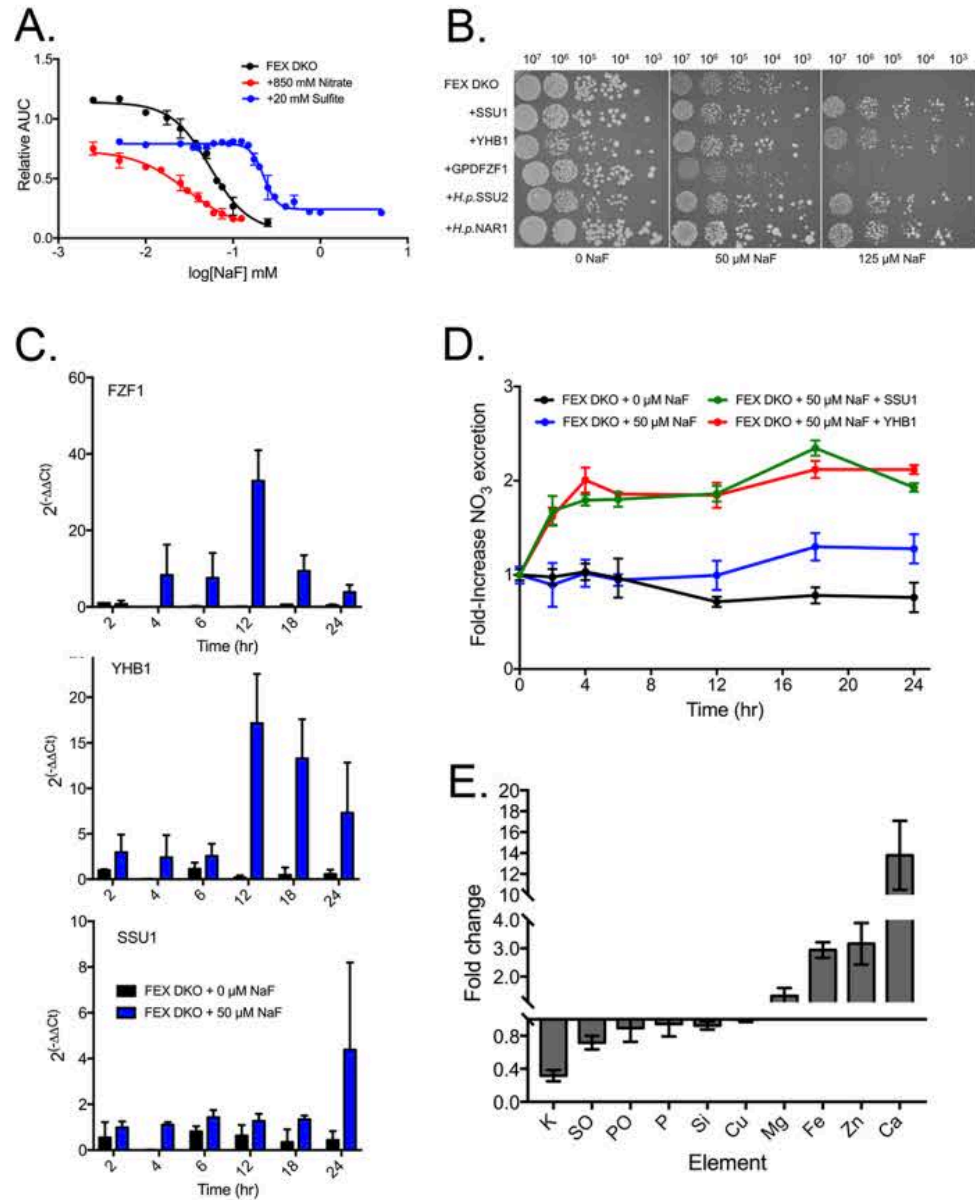
**Figure 4: Increased expression of known fluoride targets and their concurrent resistance to fluoride in yeast growth.** Liquid growth assay of FEX DKO cells +/- pRS426GPD plasmids over a 24-hour period for proteins involved in (A) oxidative stress response and (B) glycolysis.

While these seven proteins may still be targeted by fluoride *in vivo*, their individual overexpression does not confer significant rescue to fluoride toxicity. It could be that these genes are not induced to high enough levels to show an effect, or that fluoride has many protein targets. The genes that provide partial rescue from fluoride toxicity (IPPI1, PHO87, PHO90, SSU1, and YHB1), are likely to be involved in a broader mechanism of resistance than simply binding to fluoride.

***Fluoride induces the nitrosative stress response*** - We sought to determine how YHB1 and SSU1 confer improved resistance to fluoride. Yhb1p and Ssu1p are both part of the nitrosative stress response pathway that converts the highly toxic nitric oxide into the more chemically inert nitrate, and then exports that nitrate from the cell. However, Ssu1p has a second function; it is also responsible for excreting sulfite. To determine if improved fluoride resistance is associated with nitrate and/or sulfite excretion by Ssu1p, we explored the toxicity of nitrate or sulfite in combination with fluoride. Nitric oxide is a gas, and cannot be readily added to the media at increasing concentrations. At the given IC<sub>25</sub> concentrations for sulfite and nitrate (20 mM and 850 mM, respectively), addition of fluoride had contrasting effects (Fig. 5A). Sulfite - a known antioxidant - had a protective effect, shifting the IC<sub>50</sub> of fluoride from 50 to 200 μM. Conversely, addition of nitrate shifted the IC<sub>50</sub> down to 30 μM. While sulfite protects from fluoride toxicity, nitrate adds to the toxicity. Given that overexpression of SSU1 enhances fluoride resistance, it appears that the benefit of removing nitrate outweighs the cost of excreting sulfite.

To further explore the combined toxicity of fluoride and nitrate, we tested whether other proteins that excrete nitrate also improve fluoride resistance. There are no known yeast channels in *S. cerevisiae* that excrete only nitrate; however, the yeast strain *H. polymorpha* expresses two channels that excrete nitrate along with either sulfite or nitrite (Cabrera *et al.* 2014). The *H. polymorpha* gene SSU2, a sulfite/nitrate exporter, has 44% sequence similarity to *S. cerevisiae* SSU1, while the *H. polymorpha* nitrate/nitrite exporter NAR1 has 41% similarity to *S. cerevisiae* SSU1. Inducing SSU2 or NAR1 in FEX DKO yeast resulted in increased fluoride resistance, depending on the copy number (data not shown). Adding the *S. cerevisiae* SSU1 promoter to *H. polymorpha* NAR1 and SSU2 resulted in the same degree of fluoride rescue as overexpression of YHB1 and SSU1 (Fig. 5B). In general, higher expression of any protein that removed intracellular nitrate also provided partial rescue from fluoride toxicity.

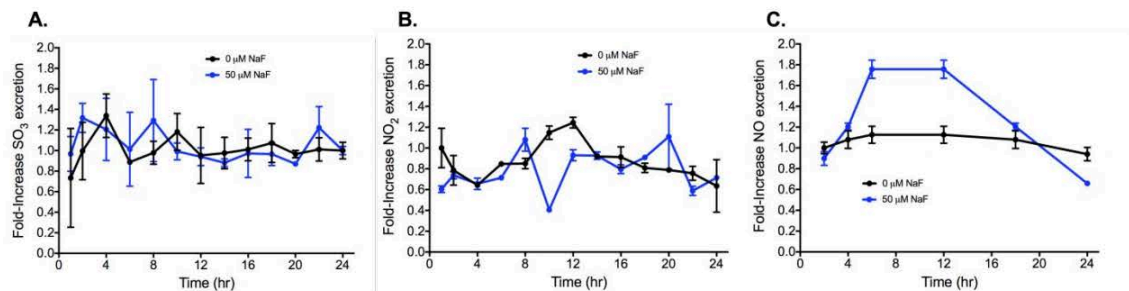
The presence of an intact promoter is essential for improved fluoride resistance in both *H. polymorpha* and *S. cerevisiae* nitrosative stress response genes. The SSU1 promoter is recognized by the five-zinc finger protein Fzf1p, a transcription factor that induces YHB1 and SSU1 expression during nitrosative stress (Sarver and DeRisi 2005). The necessity of the promoter for fluoride rescue suggests that Fzf1p is activated during fluoride exposure. Overexpression of FZF1 conferred only modest rescue from fluoride, suggesting it may already be induced to optimal levels.



**Figure 5: Nitrosative stress in fluoride-treated FEX DKO yeast.** (A) Liquid growth assay of the combined effect on growth of yeast with fluoride and the IC<sub>25</sub> of nitrate (850 mM) or sulfite (20 mM). (B) Serial dilution of FEX DKO cells ± pRS426 plasmids over a 24-h period. For *H. polymorpha* genes SSU2 and NAR1, both plasmids contain the promoter region upstream of the *S. cerevisiae* SSU1 gene. (C) RT-qPCR of FZF1, YHB1, and SSU1 gene expression over time, compared with 1 h growth in YPD and using actin as a housekeeping gene. (D) Efflux of extracellular nitrate in cells over time. (E) Fold change in intracellular ions of FEX DKO + 50 μM NaF after 24 h using ICP-MS. SO and PO are oxidized sulfur and phosphorus, respectively.

To test whether FZF1, YHB1, and SSU1 are induced under fluoride exposure, expression of each was monitored using RT-qPCR (Fig. 5C). We observed significant induction of FZF1 after 4-6 hours, and further induction after 12 hours. YHB1 was induced after 12 hours. We did not detect significant induction of SSU1 until 24 hours. However, other labs have also reported slight to moderate induction of SSU1 under conditions of nitrosative stress, which indicates that small increases in total SSU1 copy number can have compounding effects on cellular resistance to nitrosative species (Sarver and DeRisi 2005; Nardi *et al.* 2010; Hennicke *et al.* 2013).

These results suggest that fluoride induces nitrosative stress in FEX DKO yeast. Nitrosative stress is mediated by the conversion of nitric oxide into nitrate, and the subsequent excretion of nitrate from the cell. To determine whether fluoride was activating the nitrosative stress pathway and subsequent excretion of nitrate from cells, we used a colorimetric assay to monitor levels of nitrate in solution. We observed a 1.5-fold increase in extracellular nitrate after fluoride exposure starting at 12 hours, in agreement with the RT-qPCR data that showed induction of nitrosative stress response genes at a similar time (Fig. 5D). Overexpression of either YHB1 or SSU1 resulted in twice the levels of extracellular nitrate. Extracellular nitrite and sulfite concentrations did not change during fluoride exposure (Fig. 6). However, intracellular nitric oxide increased from 6 to 12 hours, before returning to baseline. An initial increase in intracellular nitric oxide and a later increase in extracellular nitrate is consistent with the hypothesis that the nitrosative stress response pathway is activated under fluoride exposure.

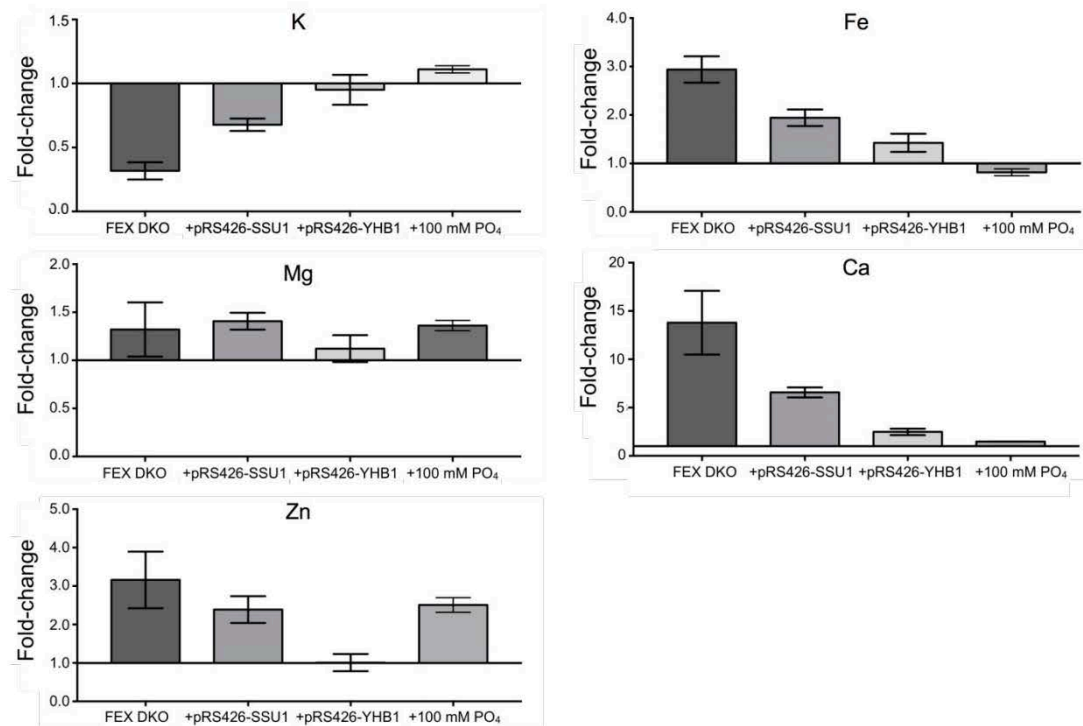


**Figure 6: Fold-change in ion concentration** over time of (A) extracellular sulfite, (B) extracellular nitrite, and (C) intracellular nitric oxide.

Nitrosative stress, while typically reported to correlate with oxidative stress, has also been reported with metal stress (Wysocki and Tamas 2010; Sahay and Gupta 2017). Fluoride has been shown in mammalian cells to disrupt metal homeostasis (Zerwekh *et al.* 1990). To test whether fluoride causes intracellular metal imbalance in FEX DKO yeast, we monitored intracellular the metal concentration using inductively coupled plasma mass spectrometry (ICP-MS). After 24 hours of fluoride exposure, the alkaline earth and transition metals magnesium, iron, zinc, and calcium increased in concentration (Fig. 5E). We also observed a decrease in intracellular potassium, which is a stress-signaling ion. One function of potassium is to regulate the membrane potential required for nutrient transport, and therefore the imbalance in intracellular metals may be linked to loss of potassium (Navarrete *et al.* 2010). The greatest change in concentration upon fluoride exposure was intracellular calcium, which increased by 15-fold. This is consistent with several reports also demonstrating an increase in calcium influx during stress, including hypertonic shock, ethanol, and alkaline stress (Bonilla and Cunningham 2002; Courchesne *et al.* 2011; Wang *et al.* 2011; Cyert and Philpott 2013). However, it is somewhat inconsistent with our data monitoring of cytoplasmic calcium using the Indo-1 dye, in which we only see a two-fold increase upon fluoride addition (Fig. 1E). This inconsistency is most likely attributed to both the increased sensitivity of ICP-MS, and that Indo-1 only monitors free, cytoplasmic calcium while ICP-MS measures total cellular calcium. ICP-MS does not differentiate between bound and unbound atoms, so we cannot determine if these metals are in complex with fluoride.

As the nitrosative stress response pathway has been shown to occur during metal stress, we hypothesized that YHB1 and SSU1 can reduce metal influx. Therefore, we also monitored intracellular metal ions in FEX DKO yeast in the presence of NaF when either YHB1 or SSU1 was overexpressed (Fig. 7). Magnesium was the only ion whose concentration was not significantly affected by overexpression of SSU1 or YHB1. Overexpressing either SSU1 or YHB1 reduced the efflux of potassium by 3-fold in SSU1, and to baseline in YHB1. Similarly, there were 2-3-fold less influx of transition metals when overexpressing SSU1, and almost no influx when overexpressing YHB1. This rescue could either be direct or indirect; YHB1 and SSU1 could work to excrete metal complexed with nitric oxide, or overexpressing YHB1 or SSU1 could counteract a stress effect upstream of metal imbalance.

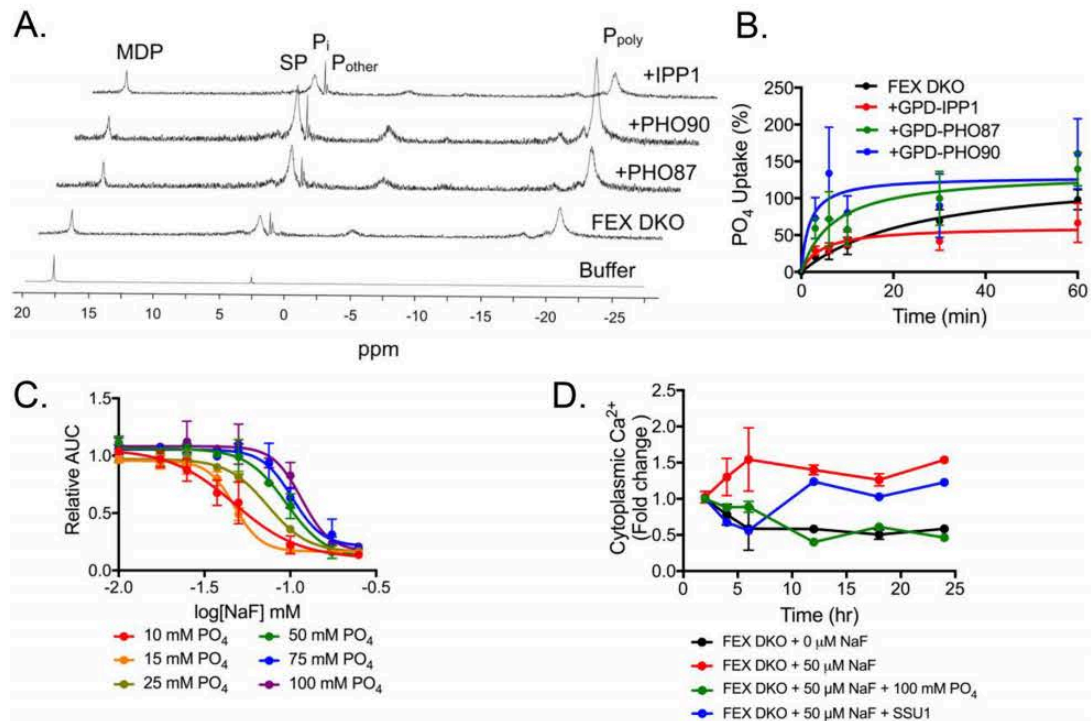




**Figure 7: ICP-MS data for FEX DKO after 24 hours growth** in YPD + 50  $\mu$ M NaF with either no plasmid, pRS426-SSU1, or pRS426-YHB1. Data is represented as fold-change in intracellular ion concentration compared with FEX DKO grown in YPD for 24 hours.

***Intracellular orthophosphate partially rescues fluoride toxicity in a concentration-dependent manner***

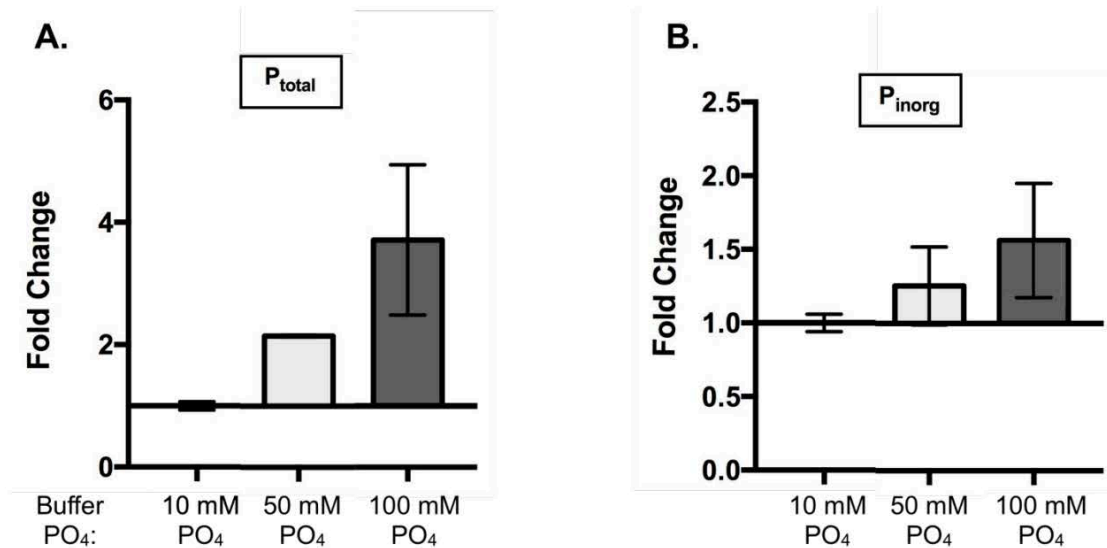
- The overexpression screen identified PHO87, PHO90, and IPP1 as genes conferring partial rescue to fluoride toxicity. PHO87, PHO90, and IPP1 are all involved in phosphate homeostasis. Pho87p and Pho90p are constitutively expressed transmembrane proteins that act as symporters of orthophosphate and hydrogen (Samyn and Persson 2016). Ipp1p is a cytoplasmic protein that converts pyrophosphate into orthophosphate (Kornberg 1962). Given these activities, overexpression of IPP1, PHO87 or PHO90 are each predicted to increase the intracellular orthophosphate concentration. To test this hypothesis, <sup>31</sup>P NMR was used to monitor total phosphate levels in FEX DKO yeast (Fig. 8A). Overexpression of either PHO87 or PHO90 increased total phosphate levels, by 4- and 6-fold, respectively. Total phosphate levels did not change upon IPP1 overexpression, but a higher fraction of the total cellular phosphate was found as orthophosphate.



**Figure 8: Intracellular phosphate in FEX DKO with high copy plasmids.** (A)  $^{31}\text{P}$  NMR spectra of yeast after 12 h growth. The peaks are (from bottom to top) buffer alone, FEX DKO, and FEX DKO with a pRS426GPD promoter expressing PHO87, PHO90, or IPP1. (B)  $^{32}\text{PO}_4$  influx assay measuring the rate of phosphate import over 60 min. (C) Liquid growth assay of cells grown in synthetic minimal media over 24 h in increasing fluoride, with the noted final concentration of phosphate present in the media. For all assays, buffer is kept at pH 6.5. (D) Fold change in the concentration of cytoplasmic calcium over time, assed using Indo-1.

The increase in total phosphate observed upon overexpression of PHO87 or PHO90 is most likely due to their function in orthophosphate import. To test this directly, we assessed the rate of phosphate import using radiolabeled phosphate uptake. Over a 1-hour timespan, there was a higher influx of phosphate in cells overexpressing PHO87 or PHO90 compared with IPP1 or FEX DKO alone (Fig. 8B). PHO90 had the highest rate of import, consistent with cells having the largest total phosphate concentrations. As overexpression of PHO90 also resulted in the greatest rescue from fluoride toxicity, we hypothesized that the rescue may directly correlate with the higher orthophosphate concentration.

The connection between greater intracellular orthophosphate and reduced fluoride toxicity suggests that higher orthophosphate alone may provide some rescue from fluoride toxicity. The overexpression screen and concurrent growth assays were conducted in YPD, which contained approximately 10 mM PO<sub>4</sub> at pH 6.5, as measured using malachite (data not shown). To assess whether an increase of orthophosphate alone is sufficient to rescue from fluoride toxicity, we performed growth assays in increasing phosphate up to 100 mM while maintaining pH at 6.5. An increase in phosphate from 10 mM to 100 mM shifted the fluoride IC<sub>50</sub> from 47 to 116 μM NaF, consistent with a concentration-dependent rescue by phosphate (Fig. 8C). We again used <sup>31</sup>P NMR and found that a 10-fold increase in extracellular phosphate led to about a 4-fold increase in intracellular phosphate (Fig. 9). The increase in orthophosphate corresponded with overexpression of PHO87 and IPP1, but was lower than overexpression of PHO90. This is consistent with the level of resistance, in which overexpression of PHO90 had 10-fold enhanced resistance to fluoride toxicity. These results are also consistent with the hypothesis that phosphate rescues fluoride toxicity independently of protein expression.



**Figure 9: Intracellular phosphate of FEX DKO yeast in increasing YPD-phosphate buffer.** (A) Fold change of total phosphate or (B) orthophosphate, as compared to cells grown in 10 mM PO<sub>4</sub>.

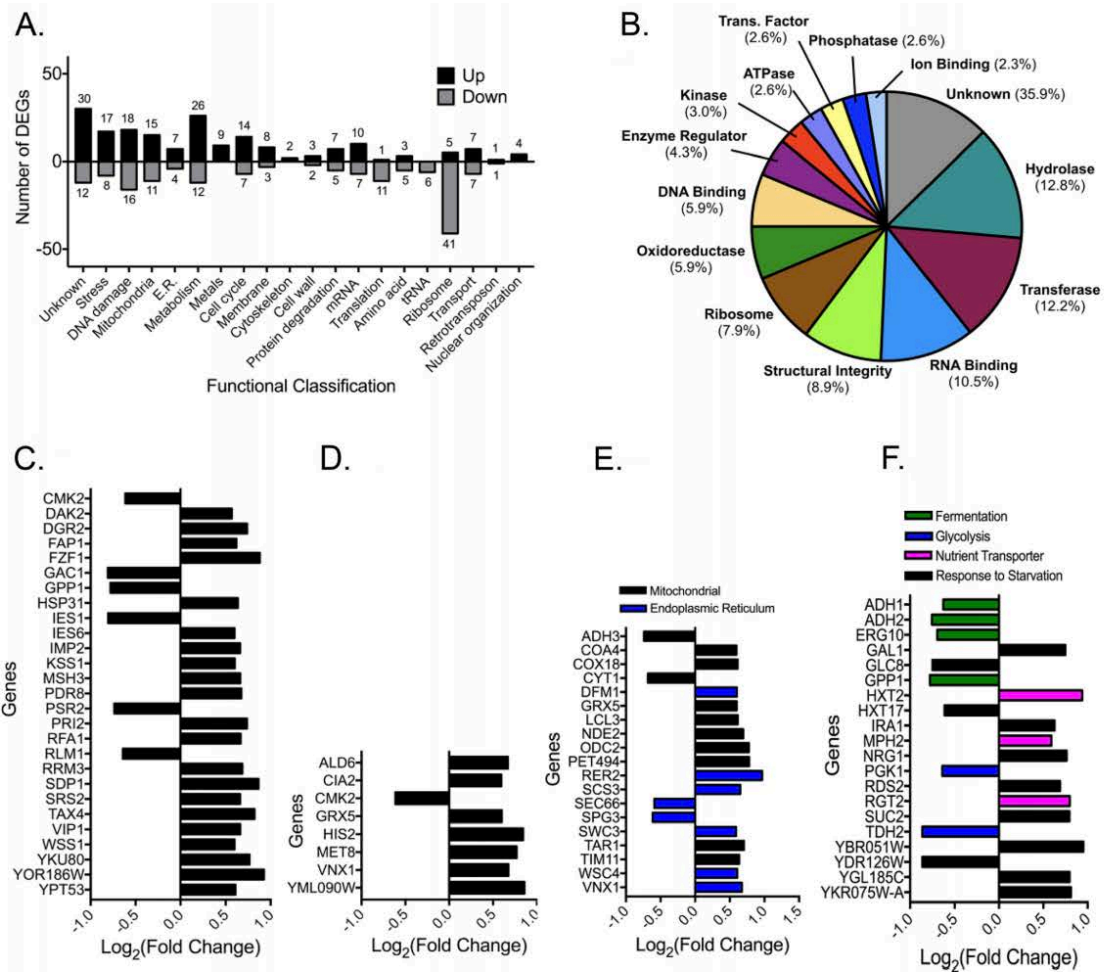
We next sought to determine whether phosphate alleviated fluoride-induced stress phenotypes. As reported above, overexpressing YHB1 and SSU1 reduced metal influx. Similarly, the addition of phosphate also lessened the efflux of potassium and influx of iron and calcium under fluoride exposure (Fig. 7). We monitored the effects of phosphate on cytoplasmic calcium, which is a signaling ion for oxidative stress (Fig. 8D). Addition of 100 mM PO<sub>4</sub> to cells resulted in a depletion of cytoplasmic calcium to the same levels as those without fluoride treatment. In contrast, enhancement of the nitrosative stress response through a high copy plasmid containing SSU1 did not alter cytoplasmic calcium. Together, these data suggest that phosphate is acting to alleviate a general stress phenotype, and is potentially doing so upstream of the onset of oxidative stress.

***Fluoride activates the starvation-induced apoptotic pathway*** - To further understand the mechanism of fluoride toxicity, including how it lowers intracellular orthophosphate and induces nitrosative stress, we examined changes in global gene expression under fluoride exposure using genome-wide RNA-Seq. Prolonged exposure to fluoride leads to eventual oxidative stress and apoptosis (Barbier *et al.* 2010). Previous studies of RNA-Seq in cells with long exposure to fluoride reported an enrichment for the induction of nonspecific stress, apoptotic and cell cycle arrest signaling genes (Pereira *et al.* 2018; Ran *et al.* 2017; Li *et al.* 2018). In order to more directly examine the mechanism of fluoride toxicity, we set out to measure gene induction after fluoride had caused toxicity, but before the cells were programmed for death. Oxidative stress signaling does not peak until 6 hours, as determined by cytosolic calcium levels and RT-qPCR, therefore, we measured gene expression of the FEX DKO yeast after 4 hours of exposure to 50 μM NaF.

The results of the RNA-Seq largely complement existing literature on fluoride toxicity (Fig. 10A). Out of 7127 genes monitored, 303 genes had over 1.5-fold change. We noted genes linked to oxidative stress, cell morphology, and general apoptotic signaling. DNA repair genes were induced, as were genes linked to translation, amino acid production and ribosomal maturation were inhibited. Consistent with our findings from the overexpression screen, there was increased expression of the nitrosative stress response transcription factor FZF1. VIP1 was also induced, which produces IP7 as the first step in the phosphate starvation response pathway (Fig. 10C).

Included among the induced genes are those linked to metal homeostasis and metalloproteins. A large fraction of the induced proteins are members of classes typically containing metals, including transferases,

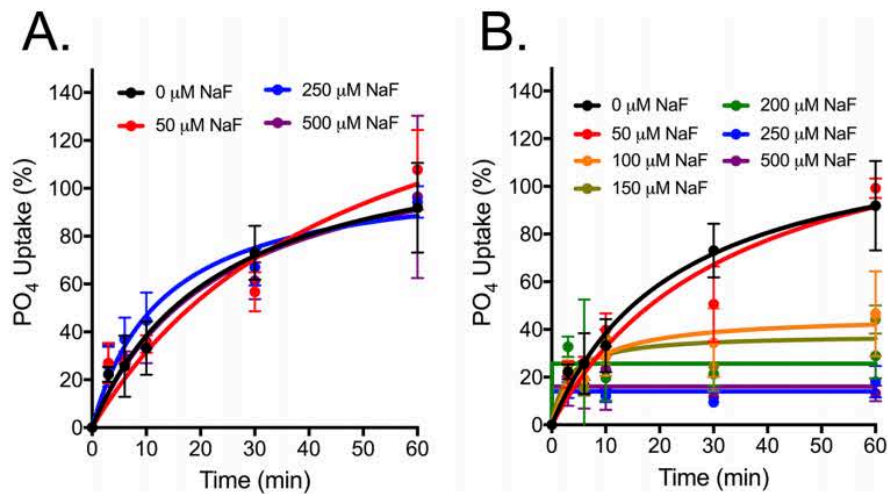
hydrolases, and oxidoreductases (Fig. 10B). Upregulated genes included those linked to Fe-S protein synthesis, calcium and magnesium regulation, and general metal resistance, as well as mRNA fragments that overlapped with copper transporter CTR1 and iron scavenger ARN2 (Fig. 10D). Upregulated genes also included those linked to mitochondrial and endoplasmic reticulum damage, a widely-reported phenotype in mammalian cells exposed to high doses of fluoride. These included genes involved in replenishing mitochondrial cytochrome c leakage and improving mitochondrial membrane integrity, as well as genes involved in endoplasmic reticulum membrane integrity and calcium storage (Fig. 10E).



**Figure 10: RNA-Seq analysis of FEX DKO treated with 50  $\mu$ M NaF for 4 h. (A) Functional classification graph of genes with more than 1.5-fold difference in expression compared with genes in FEX DKO grown in YPD for 4 h. (B) Pie chart of the protein class and function, as mapped using the Saccharomyces Genome Database gene ontology slim mapper. (C) Fold-change in expression of genes from the list of hits linked to stress response and DNA repair, (D) metal homeostasis, (E) mitochondrial and endoplasmic reticulum stress, and (F) glucose starvation.**

Compared to other RNA-Seq experiments from fluoride exposure, a unique observation in this screen was increased expression of a cluster of genes linked to glucose and nutrient starvation (Fig. 10F). Among the genes with significantly altered expression were seven involved in promoting glycolysis, as well as three sugar transporters. Previous studies that tested high extracellular fluoride suggested that fluoride directly inhibits glycolysis (Feig *et al.* 1971; Qin *et al.* 2006; Shearer and Suttie 1970). This should theoretically lead to a buildup of glucose. Instead, in the model of a FEX double-knockout yeast with low extracellular fluoride, cells appear to be scavenging glucose within four hours of fluoride exposure. This complements the phosphate starvation phenotype, and suggests an overall trend of nutrient starvation under fluoride exposure.

**Fluoride-induced stress inhibits phosphate import** - The overexpression screen identified two phosphate transporters that confer partial fluoride resistance. Similarly, three sugar transporters are induced in the RNA-Seq experiment. These observations suggest that fluoride inhibits the transport of nutrients. Several previous reports found that fluoride can inhibit glucose import (Germain and Tellefson 1986; Iwami *et al.* 1995; Rogalska *et al.* 2017). We tested for Pho87p/Pho90p inhibition by monitoring phosphate uptake under increasing fluoride (Fig. 11A).



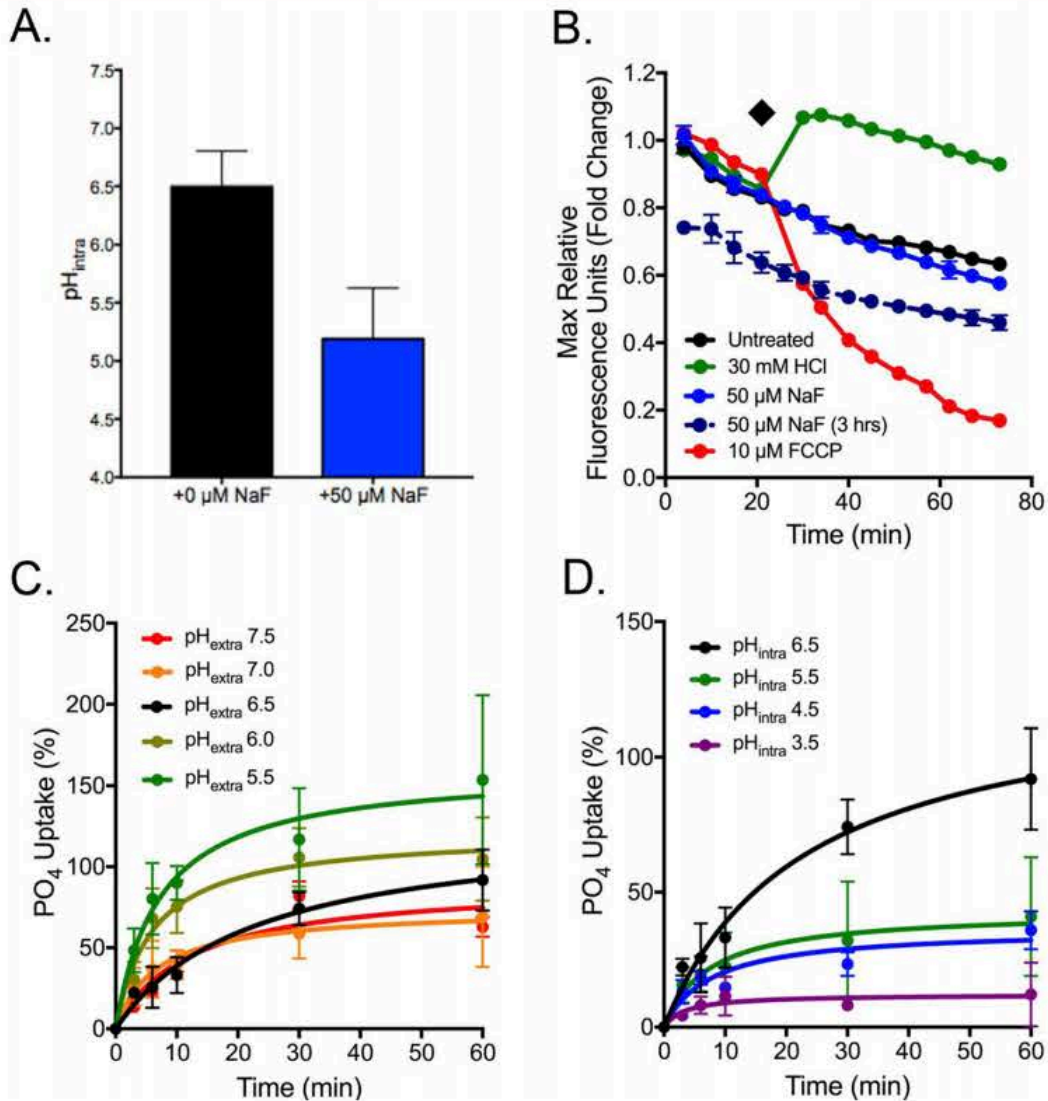
**Figure 11:  $^{32}\text{P}$  influx assay as a measurement of phosphate uptake over 60 min in 10 mM  $\text{PO}_4$  buffer at pH 6.5.** FEX DKO yeast were grown 3 h in (A) YPD or (B) NaF before transferring to  $^{32}\text{PO}_4$  and increasing NaF.

Simultaneous addition of fluoride with phosphate showed no detectable difference in phosphate uptake. However, when the cells were pretreated for two doubling times (3 hours) with fluoride, phosphate uptake was inhibited with a  $K_I$  of 90  $\mu$ M NaF (Fig. 11B). At this 3-hour timepoint, FEX DKO yeast show slower growth and decreased intracellular ATP (Fig. 1). This suggests that the inhibition of phosphate import by fluoride is indirect.

We next sought to determine what phenotype occurs at 3 hours of fluoride exposure that would arrest phosphate import. At this time point, oxidative stress signaling is just beginning to occur (Fig. 1). One major hallmark of oxidative stress is a drop in intracellular pH and consequent disruption of membrane potential (Tsai *et al.* 1997; Orij *et al.* 2011; Birben *et al.* 2012). We directly tested this by monitoring intracellular pH after 3 hours of fluoride exposure, and found that the yeast's cytoplasmic pH dropped by a full unit (Fig. 12A). We then monitored the plasma membrane electrochemical potential using the dye diS-C3, and found that while immediate exposure to fluoride had no effect on the membrane potential, 3 hours of exposure led to a destabilization consistent with intracellular acidification (Fig. 12B).

Although Pho87p and Pho90p are evolved from sodium transporters, they are predicted to be hydrogen symporters, and may be sensitized to changes in membrane potential. To determine whether this model can explain the arrest in phosphate import, we repeated the phosphate influx assay in FEX DKO yeast while changing either extracellular, or intracellular pH. Lowering extracellular pH through the addition of hydrochloric acid increased the amount of orthophosphate able to enter the cells per unit of time (Fig. 12C). Conversely, an increase in intracellular acidity through pre-incubation of cells with acetic acid, then returning cells to a neutral extracellular buffer, arrested phosphate uptake (Fig. 12D). These data collectively suggest that phosphate uptake is highly sensitized to changes in the pH gradient, such as intracellular acidification.





**Figure 12: Assessment of phosphate influx as a function of pH.** (A) Intracellular pH of FEX DKO cells after 3 h growth in YPD or 50  $\mu\text{M}$  NaF, as determined using 5(6)-CFDA. (B) Measurement of electrochemical potential of cells in either 3 h exposure to fluoride (dashed line), or immediate exposure to NaF, FCCP (an agent that causes a rapid drop in  $\text{pH}_{\text{intra}}$ ), or HCl (an agent that causes a rapid drop in  $\text{pH}_{\text{extra}}$ ). The ( $\blacklozenge$ ) indicates the point in which compounds were added for immediate exposure. (C) FEX DKO yeast grown for 3 h in YPD, before transferring to buffer with pH from 5.5 to 7.5, using HCl and NaOH. (D) Cells grown in acetic acid for 30 min to  $\text{pH}_{\text{intra}}$  of 3.5–6.5, then transferred to buffer at pH 6.5 for measurement of  $^{32}\text{PO}_4$  uptake. For further information as to the protocol, see Experimental Procedures.

## **Discussion.**

Prolonged exposure to high doses of fluoride is reported to result in many downstream effects, most notably oxidative stress, DNA damage, and metabolic arrest. These phenotypes have been attributed to a wide variety of mechanisms in the literature, most notably metalloprotein inhibition of the glycolytic enzyme enolase as well as mitochondrial respiratory proteins. Here, we report the first investigation of fluoride toxicity in a cell incapable of removing intracellular fluoride. These cells undergo growth arrest at fluoride concentrations well below those required to inhibit metalloprotein *in vitro*. Fluoride-sensitized yeast showed signs of oxidative stress, nitrosative stress, and nutrient starvation. Both oxidative and nitrosative stress are downstream phenotypes, and correlate with mitochondrial stress signaling and metal imbalance. Nutrient starvation was primarily caused by the inability of yeast to uptake phosphate and glucose upon fluoride exposure. We propose that this effect is due to the acid stress-induced disruption of the plasma membrane electrochemical gradient.

We found that overexpression of the genes IPP1, PHO87, PHO90, YHB1 and SSU1 partially rescue cells from intracellular fluoride toxicity in FEX DKO yeast. None of these proteins provide rescue in wild type background, suggesting that the mechanism of toxicity is different. Of the proteins conferring rescue to FEX DKO yeast, only IPP1p is a known target of fluoride. We found that YHB1 and SSU1 are induced upon fluoride exposure, and import of phosphate by PHO87 and PHO90 is inhibited upon fluoride exposure.

A previous study found that high copies of a genome fragment containing SSU1 rescued selenite toxicity (Perez-Sampietro *et al.* 2016). However, when SSU1 was expressed in a plasmid, it did not rescue selenite toxicity. Here we report that the SSU1 gene fragment requires its native promoter to rescue from fluoride toxicity. Likewise, YHB1 also rescues when its native promoter is included. Both SSU1 and YHB1 participate in nitrosative stress response. Nitrosative stress is linked to metal imbalance, oxidative stress, endoplasmic reticulum damage and metalloprotein inhibition. Fluoride is known to inhibit metalloproteins, damage the endoplasmic reticulum and cause oxidative stress. Increased nitric oxide has been found in the plasma of several mammalian models exposed to high levels of fluoride (Zhou *et al.* 2015; Krechniak and Inkielewicz 2005; Agalakova and Gusev 2012). Consistent with these reports, we found direct evidence of fluoride activating the nitrosative stress response inside a cell.

PHO90 overexpression provided the most efficient rescue from fluoride toxicity. It was previously shown that cells only expressing PHO90 for phosphate transport survived better in differing phosphate conditions than cells expressing only PHO87, suggesting that PHO90 is more biologically important than PHO87 (Ghillebert *et al.* 2011). Similarly, our yeast with high copy PHO90 have a higher toxicant resistance than yeast with high copy PHO87. We found that this enhanced survival correlated with greater intracellular orthophosphate concentrations.

The mechanism by which phosphate rescues fluoride toxicity remains unclear. Phosphate has many properties that could contribute. Phosphate drives forward glycolysis, inhibits the mitochondrial pore opening in yeast, chelates metals, increases ATP, and acts as a pH buffer. It is not necessarily the case that only one of these properties is responsible for rescuing from general stress. A study demonstrated that PHO80 mutants – incapable of regulating the PHO pathway – show an influx of intracellular metals similar to what we observe upon fluoride treatment (Dominguez *et al.* 1991). This supports the argument that the metal influx is due to stress and concurrent loss of membrane integrity rather than a specific attribute of fluoride.

Other labs have observed that fluoride causes disruption of mitochondrial membrane potential, the release of cytochrome c into the cytoplasm, and induction of oxidative stress (Anuradha *et al.* 2001; Kubota *et al.* 2005; Zhang *et al.* 2008; Sharma *et al.* 2008; Fina *et al.* 2014). Studies have also reported an increase in cytoplasmic calcium and endoplasmic reticulum stress, as well as inhibition of Ca<sup>2+</sup>-ATPase (Sharma *et al.* 2008; Dominguez *et al.* 1991; Boink *et al.* 1994; Xu *et al.* 2007; Murphy and Coll 1992). Our RNA-Seq data complements these findings; however, multiple labs have argued that these effects are all independent from each other and result from the high affinity of fluoride to metalloproteins, which is in the millimolar range. We observed the same pathways are affected at much lower concentrations of fluoride *in vivo*, suggesting that the inhibition may be indirect. The affected genes can be clustered into the known pathways for starvation-induced apoptosis. Mitochondrial and endoplasmic reticulum stress appear to be downstream stress effects from toxicity and may be a result of calcium influx. Supplementing cells with phosphate reduces stress signaling, further supporting that these phenotypes are downstream of nutrient starvation.

A key finding from this study is the observation that fluoride activated the indirect arrest of nutrient uptake and subsequent starvation pathway. Fluoride is known to inhibit glycolysis and drop ATP concentration. Theoretically, glycolysis inhibition should lead to an accumulation of intracellular glucose.

Instead we observed that cells demonstrate a glucose starvation phenotype. Similarly, a recent study in rat liver also showed an induction of glucose scavenger genes during fluoride exposure (Pereira *et al.* 2018). We also noted an arrest in phosphate uptake upon fluoride exposure. However, RNA-Seq did not show significant induction of the phosphate scavenging proteins in the PHO pathway, with the exception of VIP1. Most likely, metabolic and cell cycle arrest decreased phosphate usage, so total phosphate was not depleted sufficiently within four hours to trigger a substantial phosphate starvation response.

We observed that lowered intracellular pH arrested phosphate import. Similar effects are seen in mammalian cancer cells, where the acidification of the outside of the cells results in a change to membrane potential (Kato *et al.* 2013; Yang and Brackenbury 2013). In cancer cases, proteins are upregulated that can function in acidic pH. In yeast, the two inducible high-affinity phosphate transporters, PHO84 and PHO89, have acidic and alkaline pH optima, respectively. This suggests that when the PHO pathway is active, the pH gradient is disrupted. Given that a wide variety of stressors lead to intracellular acidification, phosphate import would be inhibited under many stress conditions.

In summary, yeast lacking fluoride exporters underwent a toxicity phenotype under fluoride exposure distinct from those previously reported at substantially higher fluoride concentrations. These hyper-sensitive yeast underwent metabolic arrest, oxidative stress, and nitrosative stress upon fluoride exposure. The subsequent influx of metals was mitigated by both heightened phosphate and the nitrosative stress response. Fluoride arrested phosphate import and triggered the nutrient starvation apoptotic pathway. Phosphate import was heavily influenced by intracellular acidification, a hallmark of general stress. As such, we hypothesize that the arrest in phosphate import and subsequent nutrient starvation during fluoride stress is caused by the indirect disruption in plasma membrane integrity.

## Chapter 3: Mutants of *Saccharomyces cerevisiae* are hardened to fluoride through intracellular acidification and nutrient sharing

*This section is a subset of a manuscript in preparation for publication. The figures and manuscript were prepared by myself and by Scott A. Strobel.*

### **Abstract:**

Fluoride is both naturally present throughout the environment and added as a supplement to drinking water. Many organisms have evolved resistance mechanisms to combat high doses of fluoride, most notably transmembrane fluoride exporters. Here we report generating 81 fluoride-resistant mutants of *S. cerevisiae* lacking fluoride exporters. The majority of mutants show a cell clustering phenotype similar to flocculation that confers survival, presumably through nutrient sharing and community resistance. The mutants also demonstrate increased intracellular acidification and lower intracellular fluoride accumulation. Given that fluoride can pass through membranes as HF, we propose that these mutants use intracellular acidification as a protein-free method of fluoride export.

### **Introduction:**

As the thirteenth most abundant element of the earth's crust, fluoride can be found throughout the air, soil, and water. It is at highest concentrations in areas with marine sedimentation, industrial waste, and volcanic activity (Garcia and Borgnino 2015). For the past eighty years, fluoride has been added to the water of many developed countries due to its benefits in oral health. Exposure to a small amount of fluoride reduces dental caries, inhibits bacterial plaque formation, and stimulates bone proliferation (Marquis 1995; Lau and Baylink 1998). While low fluoride exposure can improve overall bone health, there is no evidence of an organism requiring fluoride. Furthermore, high levels of fluoride cause widespread adverse effects.

Heightened fluoride exposure causes a range of toxic effects on organisms. In multicellular organisms, these effects include inflammation, skeletal defects, and tissue damage (NRC 2006; Everett 2011; Chen *et al.* 2018). Within single cells, fluoride causes oxidative stress, DNA stress, nitrosative stress, and cell cycle arrest (Zhang *et al.* 2008; Barbier *et al.* 2010; Inkielewicz-Stępnik and Knap 2012). One of the most widely reported phenotypes of fluoride exposure is metabolic inhibition, marked by a decreased rate of glycolysis and reduced concentration of ATP (Guminska and Sterkowicz 1976). Fluoride also damages the nucleus,

mitochondria, endoplasmic reticulum, cell membrane, and ribosomes after chronic exposure (Vesco and Colombo 1970; Batenburg and van der Bergh 1972; Susheela and Jain 1986; He and Chen 2006; Sharma *et al.* 2008). The exact mechanism of these effects is unknown.

Hydrofluoric acid's entry into the cell is due to its properties as a weak acid. Fluoride has the highest pKa of any halide at 3.2. As such, in an acidic solution a significant proportion of fluoride is found as HF. HF freely diffuses across biological membranes into the cytoplasm, which for most organisms is around neutral pH. Upon deprotonation, the fluoride becomes trapped, generating a gradient and resulting in intracellular fluoride accumulation. Consequently, cells are most sensitive to fluoride in an acidic environment, where substantial concentrations of HF are found.

In 2012, it was discovered that bacteria induce the expression of genes involved in fluoride export, DNA repair, and metabolic regulation upon exposure to fluoride (Baker *et al.* 2012). One of the genes induced encodes the fluoride channel Fluc. Fluc allows selective passage of fluoride ions across the plasma membrane, and deletion of Fluc results in over 200-fold increased sensitivity of bacteria to fluoride (Baker *et al.* 2012). Fluc has over 8,000 homologs across archaea, prokaryotes, and eukaryotes (Li *et al.* 2013). Our lab recently reported that *S. cerevisiae* lacking both copies of the fluoride exporter (FEX), along with having over 1000-fold sensitivity to fluoride, showed signs of nutrient starvation in response to fluoride. Subsequent RNA-Seq experiments suggested an upregulation of nutrient scavenger proteins during fluoride exposure, particularly for glucose (Johnston and Strobel 2019). Similarly, other labs have found cell lines particularly resistant to fluoride that have mutations in either genes or promoters of genes linked to metabolism and nutrient uptake (Ma *et al.* 2016; Liao *et al.* 2017). These fluoride-resistant organisms are either found through serendipity, or are generated in labs using various mutational screens.

Mutational assays have been previously used to investigate important genes for toxicity resistance, with differing success. As DNA mutations occur randomly, the assay has a high risk for generating false positives. In the context of fluoride research, mutational screens can result in fluoride-resistant organisms with anywhere from tens to thousands of altered genes (Everett *et al.* 2009). Typically, scientists look through these vast lists for genes that have already been attributed with fluoride resistance. For example, a fluoride resistant bacterial strain was found to have an operon containing an extra fluoride channel, linked to other known fluoride targets involved in metabolism and DNA repair (Liu *et al.* 2017). While this approach has

been able to generate useful data, it remains challenging to find new genes previously unlinked to fluoride amongst the vast dataset of mutations.

Here, we report generating 81 fluoride-hardened *S. cerevisiae* using a mutational screen. The large number of fluoride-hardened strains allow us to examine conserved mutations and overall trends within the dataset.

### **Experimental Procedures:**

For further experimental protocols and a more detailed description of methods, please see the Supplementary Information Materials and Methods section.

2.1 Strains and reagents: A list of reagents are available in SI Materials and Methods. Yeast used in this study are the same as described previously; wild type is BY4741, and the FEX DKO strain was previously generated using the hphMX4 and kanMX6 resistance cassettes (*MATa his3Δ1 leu2Δ0 ura3Δ0 FEX1A::kanMX6 FEX2A::hphMX*).

2.2 Generating fluoride-hardened mutants: Yeast gained a resistance to fluoride through four independent methods. Each method involved exposing a FEX double-knockout strain of *S. cerevisiae* to a mutagen (either UV or NaF), followed by screening the yeast for retained fluoride resistance.

2.2a UV method: FEX double-knockout yeast cells were grown for 48 hours on YPD-agar plates in a 30°C incubator. These plates were exposed directly to UV light using a UV lamp (UVP UVGL-58 Handheld at 0.12 Amps, 254/365 nm UV) for 20 minutes. Then the cells were replica plated onto fresh YPD-agar plates and allowed to grow 24 hours at 30°C. Individual colonies were tested for fluoride resistance by initial screening on 500 μM and 5 mM NaF-agar plates, followed by liquid growth assays to attain the IC<sub>50</sub>'s of each colony.

2.2b Spontaneous method: FEX double-knockout yeast cells were grown for 48 hours on YPD-agar plates in a 30°C incubator. Yeast were first grown on 50 μM NaF-agar plates, then individual colonies were directly plated onto 500 μM and 5 mM NaF-agar plates. Any yeast that grew on these plates were stored on YPD-agar, and their IC<sub>50</sub> was attained using a liquid growth assay.

2.2c NaF method: FEX double-knockout yeast cells were grown for 48 hours on YPD-agar plates in a 30°C incubator. Each yeast colony was incrementally added to increasing fluoride, at the concentrations of 50, 125, 250, 500, 750, 1,000, 2,500, 5,000, 7,500, 10,000, 15,000, 25,000, 30,000, and 50,000 μM NaF. For

colonies that did not grow at higher fluoride concentration, yeast from that colony was repeatedly exposed to NaF until the colony adapted to grow on that NaF. Cells able to grow on 30 mM NaF plates were streaked onto YPD plates for storage, and their IC<sub>50</sub> was assessed using liquid growth assays.

2.2d YPD method: The YPD method is the same protocol as the NaF Method, except that in-between each fluoride plate of increasing concentration, yeast colonies are streaked onto YPD plates for 48 hours growth to allow recovery.

2.3 Sequencing analysis: Sequencing results were analyzed using a computer cluster system. The published *S. cerevisiae* genome from the *Saccharomyces* Genome Database (yeastgenome.org) was used as a reference index. DNA from FEX DKO yeast that had not been exposed to fluoride was also sent for sequencing as a control. DNA for each mutant was aligned to the reference genome and the files were restructured using BWA and SAM Tools. Picard was used to mark duplicates. Freebayes was used for calling variants. The online Variant Effect Predictor ([useast.ensembl.org/info/docs/tools/vep/index.html](http://useast.ensembl.org/info/docs/tools/vep/index.html)) was used to establish yeast annotations.

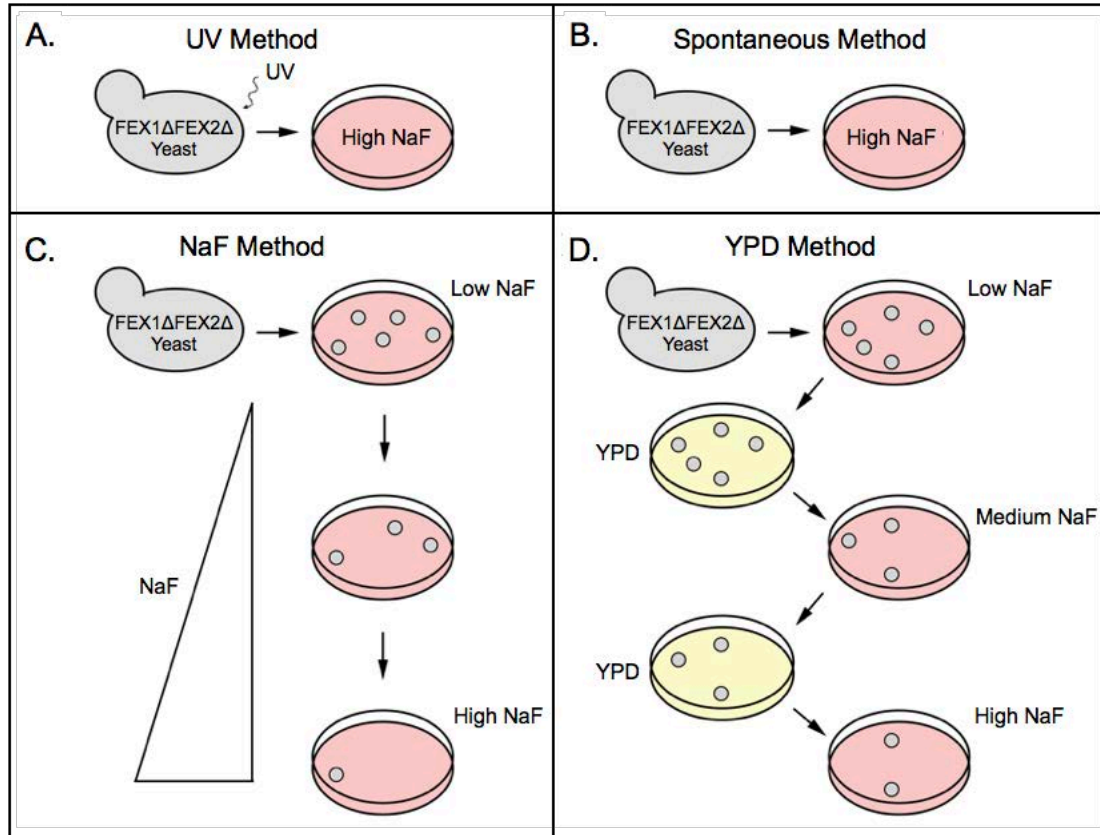
2.4 Intracellular pH: Intracellular pH was measured using the pH-sensitive dye 5(6)-carboxyfluorescein diacetate (CFDA). Unless otherwise noted, cells were grown to log-phase. Mutants differed in growth rate, so the amount of incubation time varied by mutant. Cells were washed three times in PBS (pH 7.0), and resuspended at O.D. 0.8 in 100  $\mu$ L PBS containing 50  $\mu$ M 5(6)-CFDA. Cells were placed in a 37°C water bath for 8 minutes, before being transferred to a 96 well plate. Fluorescence (492 nm excitation, 517 nm emission) was monitored using a plate reader. The fluorescence of each sample was then compared with a standard generated of yeast cells at O.D. 0.8 permeabilized in 70% ethanol for 45 minutes before being transferred to PBS at increasing pH ranging from 3.5 to 7.0. After 20 minutes, permeabilized cells were washed and placed in PBS (pH 7.0) with 50  $\mu$ M 5(6)-CFDA and placed in the 37°C water bath for 8 minutes. The pH of experimental cells was compared with the standard curve generated from the permeabilized cells. Each cell's intracellular pH was measured in triplicate.

## **Results and Discussion:**

***Generation of fluoride-hardened *S. cerevisiae**** - 81 colonies of FEX double-knockout (DKO) yeast were hardened against fluoride using four mutational assays (Fig. 1). These assays either involved the immediate



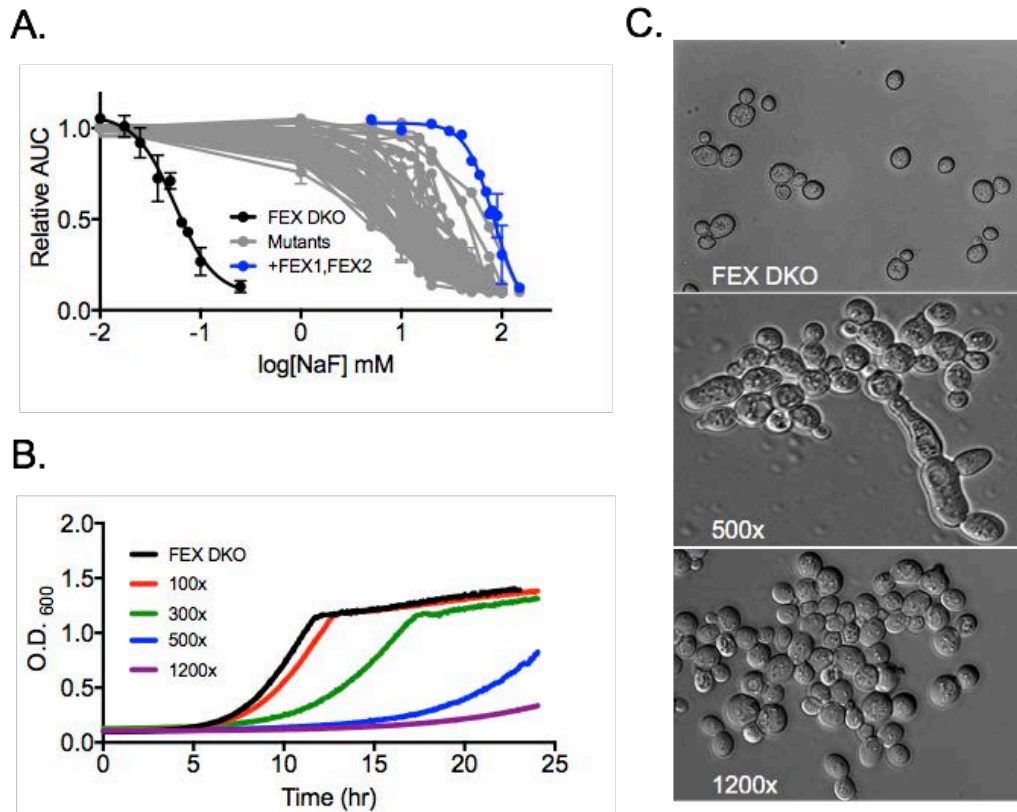
exposure of yeast to high levels of a mutagen (UV and Spontaneous Methods), or the incremental introduction of a yeast to a mutagen (NaF and YPD Methods).



**Figure 1: Overview of mutational assays.** (A) UV Method – yeast are exposed to UV light for 20 minutes, then after a 12 hour recovery step on YPD, tested for fluoride resistance. (B) Spontaneous Method – yeast grown on YPD plates are directly streaked onto 500  $\mu$ M and 5 mM NaF. (C) NaF method – yeast colonies are sequentially exposed to increasing fluoride until they can no longer grow. (D) YPD Method – yeast are grown in increments of increasing fluoride, the same as the NaF Method. In this case, yeast are given two-day recovery in YPD between each fluoride exposure.

Each technique offered unique advantages. The UV and Spontaneous Method were the fastest protocols for attaining mutants, but because of the high degree of cell stress involved, the overall rates of yeast survival and generation of fluoride-resistant mutations was low. The NaF Method was adapted from the technique put forth by the Liu lab, who generated two fluoride resistant mouse fibroblast cell lines by incrementally

increasing their exposure to fluoride (Ran *et al.* 2017). The rate of cell recovery of the NaF Method is much higher than the UV and Spontaneous Methods. However, a technique like the NaF Method could result in cells inducing genes as part of a stress response pathway, thus gaining resistance without mutating the cells. To address this concern, we also generated mutants using the YPD Method, in which yeast were allowed to recover in between fluoride exposures. This recovery step allowed cells to return to basal conditions, such that any resistance mechanism to fluoride must be retained either genetically or epigenetically for yeast to continue growing when re-exposed to fluoride. In the case of epigenetics, each resulting strain would have a similar resistance to fluoride. As this screen yielded 81 colonies with anywhere from 100 to 1200 increased fluoride resistance compared with FEX DKO, resistance is most likely due to random mutations (Fig. 2A).



**Figure 2: Overview of yeast hardened against fluoride.** (A) Liquid growth assay of either the control (FEX DKO) cells, mutants, or wild type (+FEX1 and FEX2). Rather than the typical 24-hour growth, each strain was monitored until growth plateaued. (B) Growth rate of yeast in YPD without fluoride. (C) Cell morphology under a microscope. Cells are suspended in PBS with no dye after growth into log phase.

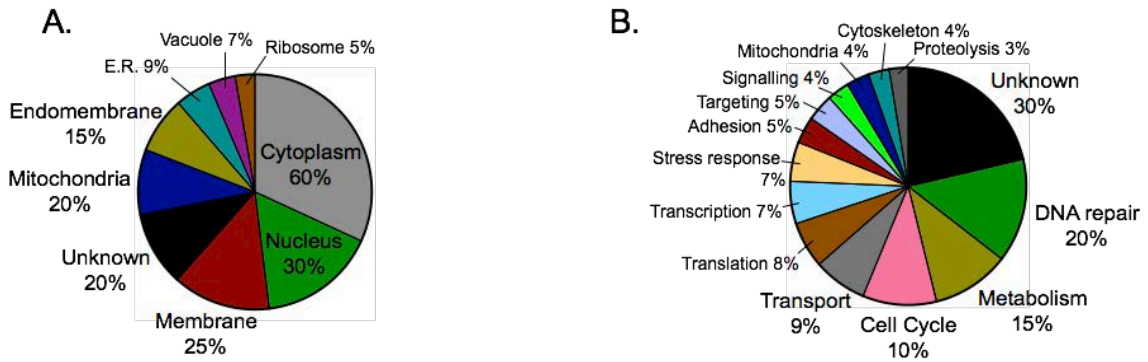
Yeast mutants often displayed slowed growth and an unusual clumping phenotype. Cells at higher resistance showed delayed growth both in entering the log phase, and a slower overall rate of log phase growth (Fig. 2B). The most fluoride-resistant mutant took three times longer to enter the lag phase than FEX DKO yeast. This is consistent with several previous studies indicating that cells with slower growth are more resistant to stressors through an unknown mechanism (Lu *et al.* 2009; Zakrzewska *et al.* 2011; Guo and Olsson 2016; Li *et al.* 2018). Yeast of higher-fold resistance also tended to cluster with one another during imaging (Fig. 2C). Some mutants ranged in size and shape, but there was no obvious correlation to resistance (data not shown). While three mutant yeast strains were around the same degree of resistant to fluoride as wild type, we were unable to attain FEX DKO mutants that were more resistant to fluoride than wild type. Given that wild type express fluoride transporters, the degree of resistance in fluoride-hardened mutants supports that the best mechanism for fluoride resistance is its removal from the cell.

***Fluoride-hardened strains are mutated in cell surface and metabolic genes*** - The 81 fluoride-hardened mutants were whole-genome sequenced to determine which mutations were most likely contributing to fluoride resistance. We also sequenced FEX DKO yeast to serve as a control, and any mutations in fluoride-hardened strains that were conserved in FEX DKO yeast were disregarded. The majority of mutations were either upstream or downstream variants, which have a higher potential to affect the expression levels of genes rather than gene function. Across all strains, we identified 1725 unique open reading frames affected by mutations, with anywhere from 19 to 106 mutations per strain, averaging 35 mutations (Table 1). As there are 6604 known open reading frames according to the yeast database, approximately one-fourth of yeast genes were affected across all 81 strains.

	UV	Spontaneous	NaF	YPD	Total
Mutants (#)	23	4	18	36	81
Fold-resistance (range)	100-1000	100-105	102-707	101-1200	100-1200
Fold-resistance (average)	233	102	283	300	274
Mutations per strain (range)	19-64	20-41	19-50	26-106	19-106
Mutations per strain (average)	31	23	34	38	35
ORFs effected per strain (range)	74-232	78-155	87-202	62-578	50-578
ORFs effected per strain (average)	115	125	128	138	129

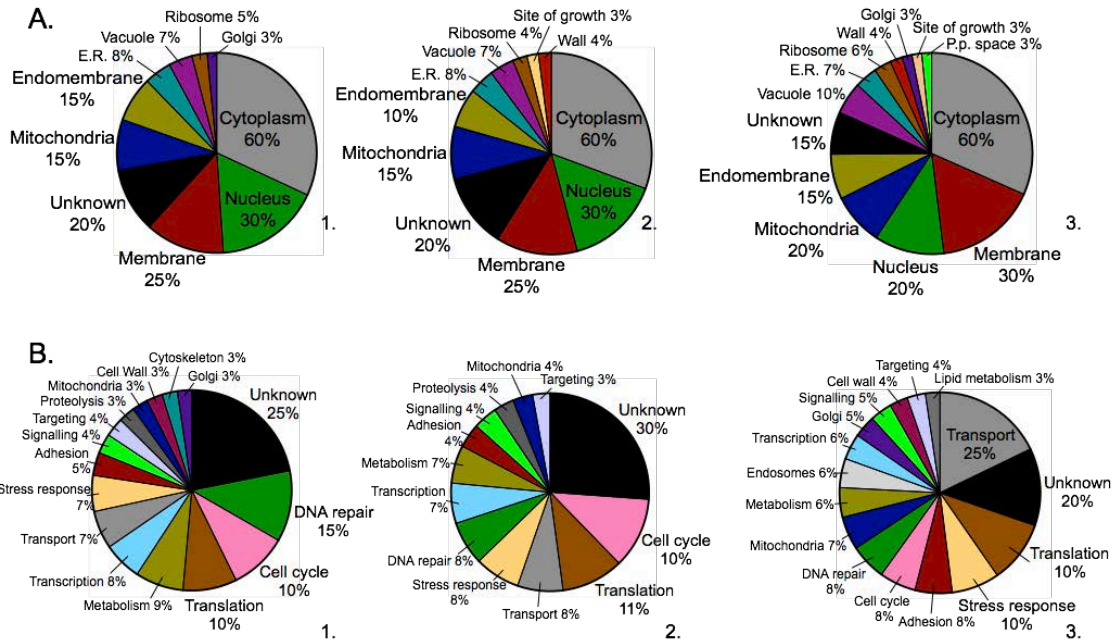
**Table 1: Summary of fold-resistance gained by assay type.** Fold-resistance was determined by the increase in IC<sub>50</sub> to fluoride compared with FEX DKO (52.67  $\mu$ M). Mutations per strain and ORFs effected were determined with bioinformatics, as outlined in Materials and Methods.

To determine whether particular organelles were important for fluoride resistance, gene ontology mapping was used to plot the percent of total genes in the mutation list whose corresponding proteins are known to localize with an organelle. The *Saccharomyces* Genome Database (SGD) was utilized for analysis as it specializes in the yeast genome. The mapping tool constructs lists of genes by annotated organelle localization, in which a gene is included for every organelle that the gene has either been found in or predicted to be in based on sequence. Therefore, the values in the pie chart add to greater than 100%. For simplicity, any organelle whose list of mutants made up less than 3% of the total list was excluded from the chart. Amongst the 6604 known open reading frames of the whole yeast genome, approximately 60% of corresponding proteins are cytoplasmic, 30% are in the nucleus, and 25% in a membrane (Fig. 3A).



**Figure 3: Gene ontology map for the yeast genome**, generated using the SGD Gene Ontology Slim Mapper tool. (A) Conserved components and (B) conserved processes for all 6604 known open reading frames in the yeast genome. In generating these pie charts, any component representing less than 2% of mutations, or any process representing less than 3% of mutations, was excluded from the list.

In all 1725 mutated open reading frames across the 81 mutants, the distribution of genes per organelle appears largely consistent with the inherent genome abundance (Fig. 4A). The greatest difference in mutations relating to organelles was the Golgi, which had three times greater enrichment. The 862 various open reading frames affected across the 27 strains with more than 200-fold increased resistance to fluoride had a mostly similar distribution. Across the 127 open reading frames impacted in the four strains with over 700-fold resistance, proteins associated with membrane function are enriched by 5%. As a whole, there was not a clear pattern for mutations to a particular organelle. This does not necessarily imply that organelle activities are not important for fluoride resistance; significant alterations to cellular processes can be made through a single mutation. Also, many vital genes are associated with several organelle functions, so gene ontology could falsely indicate no correlation of mutations to a particular organelle.



**Figure 4: Gene ontology map of conserved mutations**, generated using the SGD Gene Ontology Slim Mapper tool. (A) Conserved components and (B) conserved processes of the yeast cell that are mutated in

mutants with (1) 100-fold or higher resistance, (2) 200-fold or higher resistance, and (3) 700-fold or higher conferred resistance to fluoride. In generating these pie charts, any component representing less than 2% of mutations, or any process representing less than 3% of mutations, was excluded from the list.

We next used gene ontology to distinguish whether particular cellular processes had conserved mutations across fluoride-resistant strains. The total *S. cerevisiae* genome is classified such that 20% of known open reading frames are involved in DNA maintenance and repair, 15% in various metabolic processes, and 10% in cell cycle and growth (Fig. 3B). Across mutants with higher resistance to fluoride, there was a two-fold decrease in abundance to DNA repair and metabolism than should be from random mutations (Fig. 4B). As fluoride is known to cause DNA damage and metabolic arrest, cells most likely face selection pressure to maintain DNA repair and metabolism as much as possible. Conversely, there was a nearly three-fold enrichment in the over 700-fold resistance strains for ion and nutrient transport genes, as well as enrichment in genes pertaining to the cell surface. This is consistent with several other reporting of fluoride-resistant organisms whose genome or RNA sequencing suggest remodeled cell surfaces in response to fluoride.

Many individual genes were heavily mutated across fluoride-hardened yeast strains, most notably those involved in cell adherence and nutrient transport. FLO9, involved in flocculation, was the most frequently mutated gene across all strains, with a total of 444 mutations (Table 2). Following this was FET4 (a plasma membrane iron transporter) at 335 mutations, and ADH6 (an alcohol dehydrogenase linked to DNA stress) and DIA1 (involved in pseudohyphal growth), both at 283 total mutations. Also among the most commonly mutated genes were IPP1 (inorganic pyrophosphatase) at 157 mutations and ERR3 (an enolase mimic) at 101 mutations, both of which are predicted targets for *in vivo* fluoride inhibition.

	Gene	Name	Total Mutations	Mutated Strains
1	YAL063C	FLO9	444	81
2	YMR319C	FET4	335	77
3	YMR316W	DIA1	283	72
4	YMR318C	ADH6	283	72
5	YAL064W	Unknown	234	73
6	YAL062W	GDH3	208	74
7	YBR011C	IPP1	157	78
8	YIL169C	CSS1	107	71
9	YIL170W	HXT12	104	70
10	YIL172C	IMA3	104	70
11	YMR323W	ERR3	101	62
12	YHL050C	TEL08L	79	73
13	YHL048W	COS8	78	73
14	YAR050W	FLO1	77	51
15	YJR151C	DAN4	67	62
16	YJR152W	DAL5	66	61
17	YLL066C	TEL12L	57	37
18	YGR055W	MUP1	56	35
19	YGR054W	eIF2A	54	34
20	YGR056W	RSC1	54	34
21	YMR322C	SNO4	52	51
22	YBL101C	ECM21	51	39
23	YMR326C	TEL13R	49	34
24	YDL245C	HXT15	47	42
25	YDL246C	SOR2	47	42
26	HRA1	HRA1	43	43
27	YAL026C	DRS2	43	42
28	YGR057C	LST7	43	33
29	YAL023C	PMT2	42	42
30	YAL024C	LTE1	42	42
31	YAL025C	MAK16	42	42
32	YDL243C	AAD4	42	41
33	YDL244W	THI13	42	41
34	YER052C	HOM3	42	42
35	YER053C	PIC2	42	42
36	YER053C-A	Unknown	42	42
37	YER054C	GIP2	42	42
38	YER055C	HIS1	42	42
39	YLL065W	Unknown	42	33
40	YLL063C	AYT1	41	33
41	YLL064C	PAU18	41	33
42	YIL047C	SYG1	40	39
43	YCR040W	MATALPHA1	39	34
44	YCR037C	PHO87	38	33
45	YCR038C	BUD5	38	33
46	YCR038W-	Unknown	38	33
47	YCR039C	MATALPHA2	38	33
48	YCR041W	Unknown	38	33
49	YCR042C	TAF2	38	33
50	YIL046W	MET30	38	37
51	YIL046W-A	Unknown	38	37
52	YIL047C-A	Unknown	38	37
53	YIL048W	NEO1	38	37
54	tF(GAA)B	tRNA-Phe	37	36
55	YCR017C	CWH43	37	34
56	YCR018C	SRD1	37	35
57	tK(CUU)C	tRNA-Lys	36	34
58	tM(CAU)C	tRNA-Met	36	34
59	YCR018C-A	Unknown	36	34
60	YCR019W	MAK32	36	34
61	snR47	SNR47	35	35
62	YDR039C	ENA2	35	35
63	YDR040C	ENA1	35	35
64	YDR041W	RSM10	35	35
65	YDR042C	Unknown	35	35
66	YDR043C	NRG1	35	35
67	YCR016W	Unknown	34	33
68	YLL067C	Unknown	34	23
69	tS(AGA)D3	tRNA-Ser	33	33
70	YDR419W	RAD30	33	33
71	YDR420W	HKR1	33	33
72	YDR421W	ARO80	33	33
73	YLL067W-A	Unknown	33	22
74	tD(GUC)N	tRNA-Asp	32	30
75	YAR071W	PHO11	32	32
76	YAR073W	IMD1	32	32
77	YAR075W	Unknown	32	32
78	YIL061C	SNP1	32	31
79	YIL062C	ARC15	32	31
80	YIL063C	YRB2	32	31
81	YIL064W	EFM4	32	31
82	YIL065C	FIS1	32	31
83	YNL055C	POR1	32	30
84	YNL056W	OCA2	32	30
85	YNL057W	Unknown	32	30
86	YNL058C	Unknown	32	30
87	tM(CAU)O1	IMT1	29	20
88	YIL066C	RNR3	29	28
89	YIL066W-A	Unknown	29	28
90	YIL067C	Unknown	29	28
91	YOR106W	VAM3	29	19
92	YOR107W	RGS2	29	19
93	YOR108C-A	Unknown	29	19
94	YOR108W	LEU9	29	19
95	YOR109W	INP53	29	19
96	YOR192C-	Unknown	29	20
97	YOR193W	PEX27	29	20
98	YOR194C	TOA1	29	20
99	YOR195W	SLK19	29	20
100	tS(AGA)B	tRNA-Ser	24	24

**Table 2: Summary of top 100 conserved mutations across all 81 strains.**

Four strains of mutants had at least 700-fold increased fluoride sensitivity. Between these four strains, there were 603 total mutations. The top 30 most mutated genes were each included in the top 100 genes mutated across all strains (Table 3).

	Gene	Name	Total Mutations
1	YAL063C	FLO9	20
2	YMR319C	FET4	10
3	YBR011C	IPP1	8
4	YMR318C	ADH6	8
5	YMR317W	Unknown	7
6	YMR323W	ERR3	6
7	TEL08L	TEL08L	4
8	YGR055W	MUP1	4
9	YJR151C	DAN4	4
10	ARS1333	Unknown	3
11	TEL12L	TEL12L	3
12	TEL13L	TEL13L	3
13	tF(GAA)B	tRNA-Phe	3
14	YBL101C	ECM21	3
15	YHL048W	COS8	3
16	YMR322C	SNO4	3
17	ARS1335	Unknown	2
18	YBL100W-C	Unknown	2
19	YCL054W	SPB1	2
20	YCL055W	KAR4	2
21	YCR018C	SRD1	2
22	YCR037C	PHO87	2
23	YER053C	PIC2	2
24	YER054C	GIP2	2
25	YGL080W	MPC1	2
26	YGL082W	Unknown	2
27	YIL064W	EFM4	2
28	YIL065C	FIS1	2
29	YIL066C	RNR3	2
30	YIL169C	CSS1	2

**Table 3: Summary of top 30 conserved mutations** across the four highest fluoride-resistant strains.

All four strains had mutations affecting genes IPP1 (inorganic pyrophosphatase), DAN4 (cell wall mannoprotein important for cell adhesion and iron uptake), and FLO9 (flocculation). Furthermore, they each had expanded telomere regions. Telomeres are known to shorten during oxidative stress, and the lengthening of the telomeres could potentially confer resistance during fluoride-induced oxidative stress. The telomeric region on the left arm of Chromosome VIII (TEL08L) was mutated in all four strains, suggesting that DNA near the left arm of this chromosome may be particularly important. TEL08L is located next to COS8 (an endosomal protein) and ARN2 (an iron transporter), and COS8 is independently on the list for most frequently mutated.

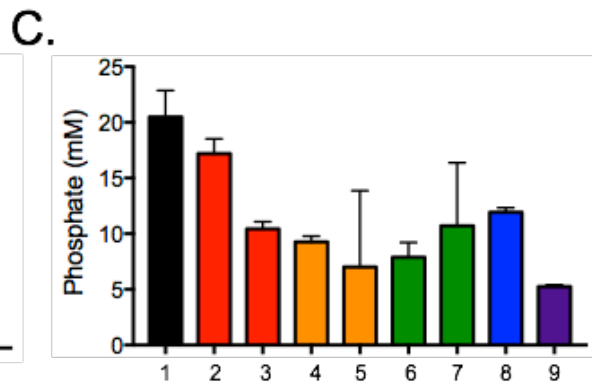
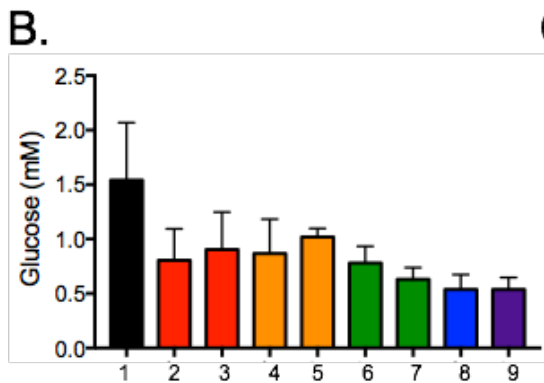
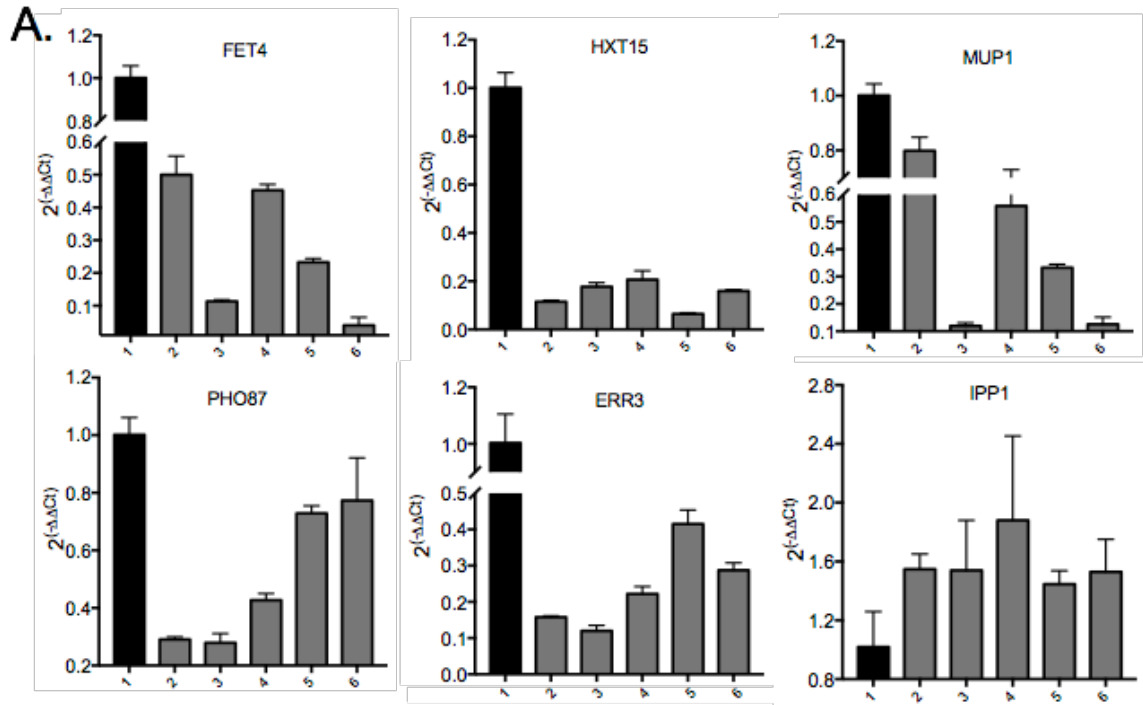
The mutants with the highest fluoride resistance were enriched in mutations relating to transport (Fig. 4B). Of the genes assigned to this category, the majority were involved in either vesicle-mediated transport, mitochondrial nutrient regulation, or single nutrient uptake, including sugars, amino acids, ATP, and cation transporters. This suggests that alterations to these pathways in particular confer fluoride resistance.

Across all mutants, the most frequently mutated genes cluster into several pathways. The majority, such as DAN4, FLO9, and DIA1, relate to the cell surface of yeast, its adherence, and ability to bud. GDH3, IPP1, ERR3, FET4, and DAN4 are each involved in metabolism and cell adaptation to nutrient starvation. Others such as the telomere length and ADH6 are linked to oxidative stress and DNA repair, which fluoride is known to trigger.

**Fluoride-hardened mutants had increased dormancy and lowered metabolism** - Mutants with higher-fold fluoride resistance showed an overall trend of slow growth. As mentioned above, multiple labs have correlated enhanced stress resistance with slow growth in single-celled organisms (Zakrzewska *et al.* 2011; Liao *et al.* 2017). Slow growing yeast typically function at lower metabolic rates. The fluoride-hardened



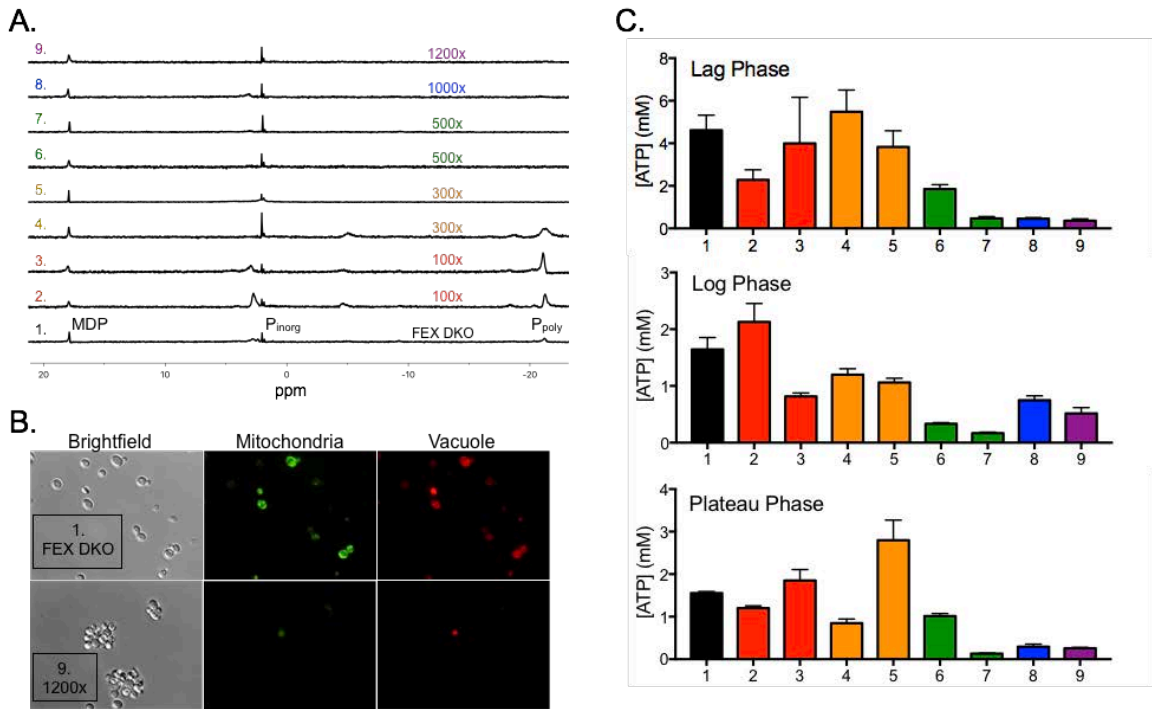
yeast isolated in this study had many mutations linked to metabolism, particularly nutrient transport, storage, and mitochondrial activity. In order to determine whether these mutations resulted in lowered overall metabolism, we examined both gene expression, as well as overall pathway activity. We first monitored gene expression of nutrient transporters and metabolic genes using RT-qPCR (Fig. 5A). For each gene, five strains were selected that featured mutations to that gene. These cells were grown to log phase, then RNA was harvested and quantified compared with actin. Overall, genes related to nutrient uptake and metabolism had lower expression compared with FEX DKO yeast. The exception to this was IPP1, which increased in expression in mutants, and PHO87, where higher-fold resistant mutants had less of a change in expression. This is consistent with our lab's previous finding that a higher copy number of IPP1 and PHO87 increases fluoride resistance. Nonetheless, other genes linked to nutrient regulation had decreased expression, suggesting that fluoride-hardened mutants had an overall decline of nutrient uptake.



**Figure 5: Gene expression and intracellular nutrient levels of fluoride hardened mutants.** (A) RT-qPCR of gene expression during log phase of (black) FEX DKO and (grey) mutants with mutations to the indicated genes. Mutants are ordered in increasing degree of resistance to fluoride. Cells were also assayed for intracellular concentration of (B) glucose and (C) for (1) FEX DKO, (2-3) 100x (4-5) 300x, (6-7) 500x, (8) 1000x, and (9) 1200x increased resistance to fluoride.

The intracellular nutrient concentration in fluoride-hardened mutants was determined using colorimetric dyes. Mutants had consistently lower glucose (Fig. 5B) and phosphate (Fig. 5C) concentrations compared with FEX DKO. This trend was consistent with the degree of fluoride resistance; that is, the higher resistant strains had the lowest intracellular nutrient levels. Together with the RT-qPCR data, the lowered intracellular nutrient levels indicate that a decline in nutrient uptake confers survival to fluoride.

As part of the investigation of intracellular nutrient levels, we also examined the organelles involved in metabolism: the mitochondria and vacuole. Several fluoride-hardened mutants, particularly those in the 300 to 500-fold increased fluoride resistance, had ATPase mutations in the vacuole and mitochondria. Vacuolar and mitochondrial ATPases are essential for maintaining pH gradient across the organelle, and mutations would theoretically inhibit organelle function. Consistent with this prediction, mutants starting in this range of fluoride resistance showed a total loss of polyphosphate, indicating inactive vacuoles (Fig. 6A). Other mutants in the range of +500-fold fluoride resistance also lacked polyphosphate, despite having normal ATPase sequences. This suggests that organelle function is decreased in the higher-fold resistant mutants, independent of ATPases.



**Figure 6: Mitochondrial activity and vacuolar activity in fluoride resistant mutants.** (A)  $^{31}\text{P}$  NMR of strains grown to log phase.  $\text{P}_{\text{poly}}$  corresponds to polyphosphate found in the vacuole during phosphate rich conditions. (B) Intracellular ATP of FEX DKO and eight mutant strains, as quantified using a luciferase-based assay. (C) Imaging of cells for mitochondrial and vacuolar activity using Rhodamine 123 and FM4-64 dye, respectively. Strains are (1) FEX DKO, (2-3) 100x (4-5) 300x, (6-7) 500x, (8) 1000x, and (9) 1200x increased resistance to fluoride.

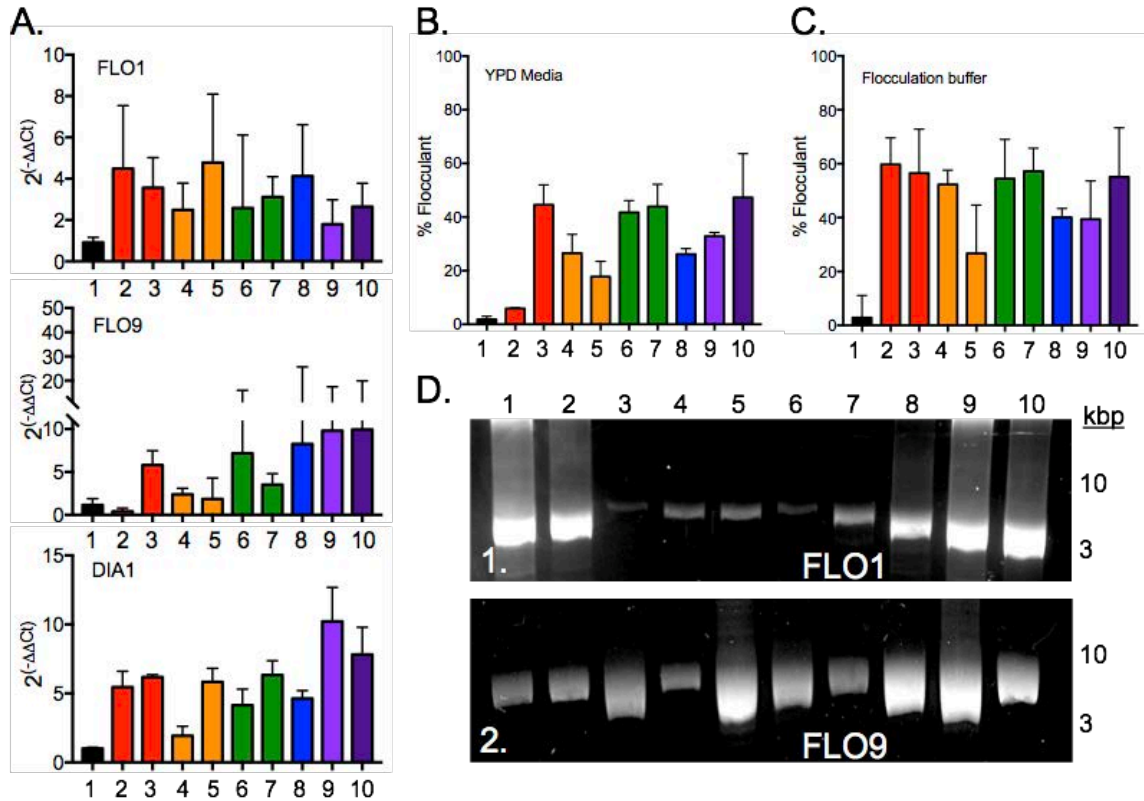
Both vacuolar and mitochondrial function were assessed using microscopy and organelle-specific dyes (Fig. 6B). These dyes incorporate into the mitochondrial or vacuolar membrane based on its active membrane potential. We assessed mitochondrial and vacuolar activity in the highest fluoride-resistant mutant. This mutant showed little to no incorporation of dye in the vacuole or mitochondria. This supports that vacuolar and mitochondrial activity is lower in the mutants, and suggests that the membrane potential is disrupted for both organelles. Similarly, we saw a decrease in intracellular ATP in the higher-fold resistant mutants, indicating a decline of mitochondrial function (Fig. 6C). Mitochondrial function was more varied in the 100 to 300-fold fluoride resistant strains, and also varied by growth phase. Nonetheless, the strains with the

highest fluoride resistance consistently had less intracellular ATP than FEX DKO, indicating an overall lower rate of metabolism. These assays have caveats however; namely, the incorporation of dyes rely on the pH gradient. Cellular acidification would also give similar results.

***Community resistance and nutrient sharing confer fluoride resistance*** - The fluoride-hardened strains had lower intracellular nutrient levels, a decrease in expression of nutrient transporters, and disrupted vacuole and mitochondria. This is consistent with our previous observation of slowed growth, suggesting cellular dormancy. Another trend that correlated with fluoride resistance was cellular aggregates, in a manner reminiscent of flocculation (Fig. 2B and 2C). From genome sequencing, we found that genes involved in flocculation and cellular adhesion were amongst the most mutated (Fig. 4). Flocculation is a process of filamentous growth through which yeast aggregate and precipitate from solution, and is very similar to a bacterial biofilm (Soares 2010). Flocculation is induced by the binding of flocculin lectin proteins of one cell to the mannose of another cell's wall. Many stimuli can activate flocculation, including nutrient starvation. During environmental stress, flocculating yeast divert nutrients to other yeast in their community to enhance overall survival. Previously, our lab found that fluoride triggers nutrient starvation response in yeast. Therefore, we hypothesized that fluoride-hardened yeast developed a nutrient scavenging phenotype similar to flocculation.

FEX DKO yeast are derivatized from *S. cerevisiae* BY4741, which is a non-flocculating strain. The BY4741 genome contains the FLO genes necessary for flocculation, although most of these genes are transcriptionally silenced. However, mutations in *S. cerevisiae* BY4741 can switch on the expression of FLO genes (van Mulders *et al.* 2009; Kim and Rose 2015; Park *et al.* 2019). Given the aggregation phenotype in some of our fluoride-hardened mutants, we predicted that a subset of mutations to flocculating genes resulted in their active transcription. The most commonly mutated genes linked to filamentous growth were FLO9, DIA1, and FLO1. FLO1 and FLO9 are lectins that function in cell adhesion, while DIA1 is linked to the other form of filamentous growth, pseudohyphal.

We monitored the expression of genes involved in filamentous growth, to examine whether mutations across strains activated their transcription *in vivo* (Fig. 7A). FLO1, FLO9, and DIA1 all had increased expression compared with FEX DKO. FLO9 and DIA1, which were more heavily mutated across strains than FLO1, also had a higher degree of correlation between increased induction and degree of fluoride resistance.



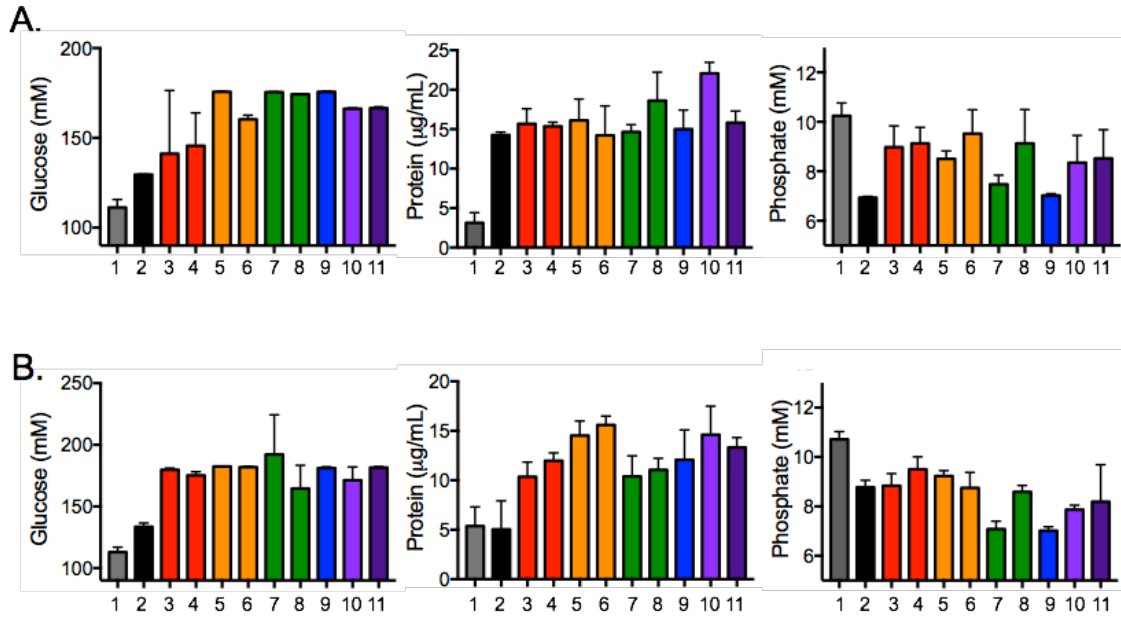
**Figure 7: Flocculation ability of fluoride-hardened mutants.** (A) RT-qPCR of strains in log phase compared with FEX DKO for FLO1, FLO9, and DIA1 genes, compared with actin. (B) Flocculation number, as determined by the percent of yeast no longer in solution after one minute at rest. Cells were either in YPD or (C) flocculation buffer, containing 5 mM calcium chloride. (D) DNA fragments of FLO1 or FLO9, generated using PCR from isolated DNA, and then ran on a 2% agarose gel. Strains are (1) FEX DKO, (2-3) 100x (4-5) 300x, (6-7) 500x, (8) 750x, (9) 1000x and (10) 1200x increased resistance to fluoride.

Flocculation can be monitored by counting the number of cells that precipitate out of solution within one minute. In regular YPD media, fluoride-hardened mutants had a flocculation number of around 20-45 (Fig. 7B). While this was much higher flocculation than FEX DKO, it still is considered weakly flocculating. Nonetheless, the effect was enhanced by switching cells into a calcium-rich buffer to promote flocculation (Fig. 7C). The degree of enhancement was inversely proportional to fluoride resistance; that is, weaker fluoride resistant strains had a higher increase in flocculation while in calcium buffer, while higher fluoride

resistant strains were able to flocculate in regular media. In all, this data confirms that the fluoride resistant mutants had enhanced flocculating capabilities compared with FEX DKO.

The strength of flocculation interactions is dependent on the flocculin protein length, and corresponding gene length. Increasing the number of tandem repeats in a FLO gene results in longer transcripts, and consequently longer FLO proteins. Higher flocculating yeast strains have longer FLO genes, resulting in greater interaction with corresponding mannose and tighter binding between cells (Gemayel *et al.* 2012; di Gianvito *et al.* 2017). We screened for changes in gene length by transcribing copies of FLO1 and FLO9 across mutants using PCR (Fig. 7D). Like RT-qPCR, the trend fluctuated for FLO1 disproportionately with fluoride resistance, but overall alterations to FLO1 resulted in longer genes. This is consistent with cells have enhanced clustering abilities. In contrast, altered FLO9 DNA was shorter compared with FLO9 in FEX DKO. Shorter flocculin proteins are more stable and resistant to changes in pH; as such, the fluoride-hardened mutants favor stable Flo9p proteins for binding (Li *et al.* 2013).

Flocculation is induced during nutrient starvation as cells subsequently form nutrient sharing communities. Typically, nutrients are released from cells into the surrounding buffer so that they can be distributed throughout the clustering cells. In order to determine whether this was occurring in the fluoride-hardened mutants, we monitored the extracellular concentrations of amino acids, phosphate, and glucose (Fig. 8A).



**Figure 8: Release of nutrients by FEX DKO and fluoride-hardened mutants.** Extracellular concentration of glucose, amino acids (measured by Bradford assay), and phosphate of cells at a final O.D. of 5.0, grown in either (A) YPD or (B) 50  $\mu$ M NaF for 24 hours.

For both amino acids and glucose, FEX DKO and mutants released nutrients into the buffer. More nutrients were present in the buffer of the mutant strains, in support of nutrient sharing. In the case of extracellular phosphate, the concentration of buffer containing cells was always lower than buffer alone, indicating that phosphate was being imported into the cells. Nonetheless, the fluoride-hardened mutants had more phosphate in the buffer than FEX DKO. We also tested the effect of fluoride on nutrient sharing (Fig. 8B). FEX DKO and mutant cells were exposed to 50  $\mu$ M fluoride for 24 hours. Extracellular glucose concentration for FEX DKO cells decreased, while remaining largely unchanged in mutant strains. Extracellular amino acid concentration decreased for both FEX DKO and mutant strains, although the mutants still retained higher extracellular concentrations. Extracellular phosphate had the opposite trend, with more phosphate appearing to enter cells upon fluoride exposure. In all, the fluoride-hardened strains had higher extracellular nutrient concentration, reminiscent of the mechanism of rescue from nutrient starvation by flocculation.

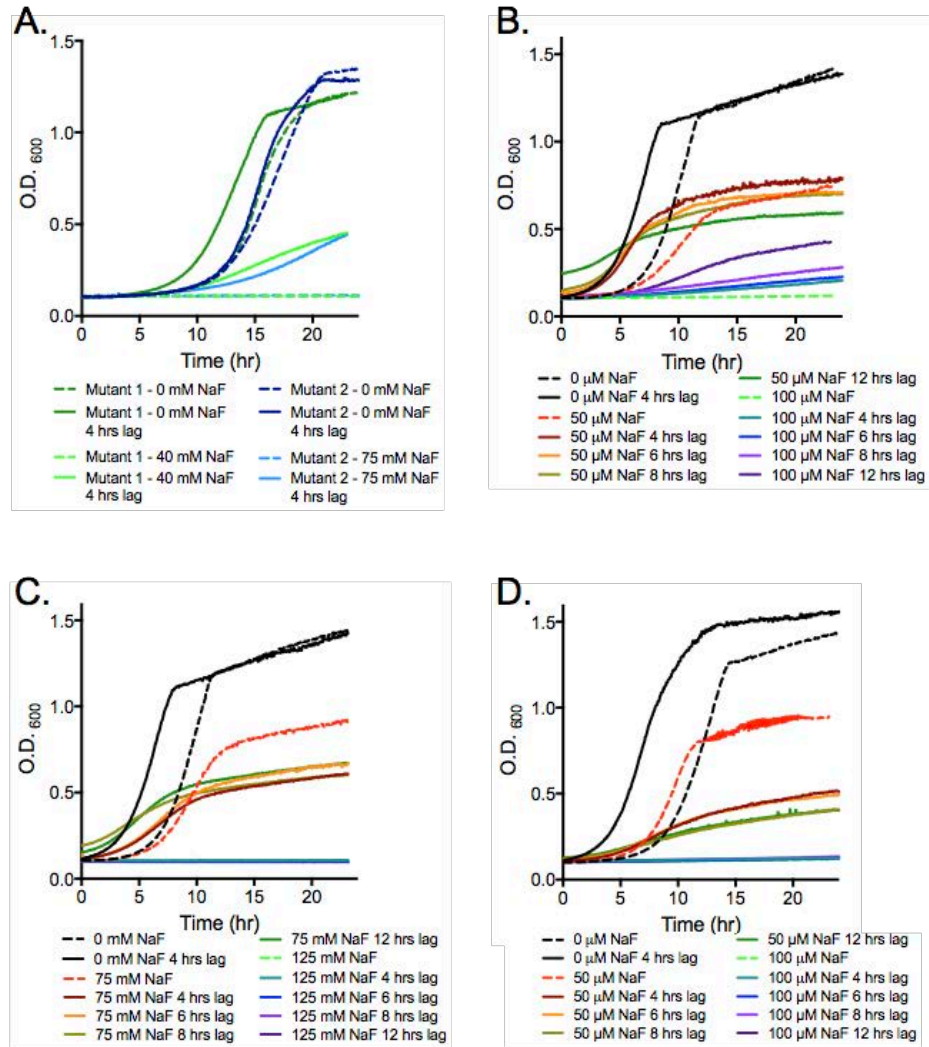
It is unclear why FLO9 is the top mutated gene instead of FLO1. Not enough is known about FLO9 to determine its use. Prior research has found that FLO9 function is responsive to changes in nutrient levels



(van Mulders *et al.* 2009). Given fluoride's ability to trigger nutrient starvation, this could lead to a more sensitive response pathway. The enrichment could also be circumstantial; FLO genes are located in the sub-telomeric regions of DNA, and we found that the telomeres were heavily mutated in fluoride-hardened strains. FLO9's subsequent mutation could be therefore be due to the mutation of TEL01L. However, FLO9 is mutated more frequently than TEL01L, which argues that FLO9 and the subsequent flocculation independently confers fluoride resistance.

***Cell sedimentation through flocculation promotes fluoride resistance*** - Some, but not all of the fluoride-hardened yeast mutants differed in their degree of resistance when grown in liquid culture instead of agar. Every mutant was selected based on its ability to grow on a high fluoride agar plate. Mutants retained their ability to grow on high fluoride agar even after being allowed to recover in YPD (data not shown). However, these yeast had widely varying IC<sub>50</sub> and IC<sub>99</sub> values between strains (Fig. 2A). Occasionally, the IC<sub>99</sub> values collected using the liquid growth assay would be lower than the concentration of fluoride in agar that the mutants were able to grow in. This suggests that many mutant yeast were losing fluoride resistance when growing in liquid.

Yeast's tendency to lose fluoride resistance in liquid correlated inversely with flocculation ability in YPD. While all yeast mutants had mutations in flocculation genes, not all strains clustered together when imaged under the microscope. We found that those that did not visibly cluster also tended to be those that lost fluoride resistance in liquid culture. Introduction of a four-hour lag step, in which cells were placed at 30°C and not shaken, resulted in cells settling to the bottom of the plates. The cells were then shaken at medium speed for 24 hours to conduct the liquid growth assay. Despite being shaken, the cells did not re-inter solution following the four-hour lag step. Consequently, these mutants grew at higher fluoride concentrations than without the lag step (Fig. 9A).

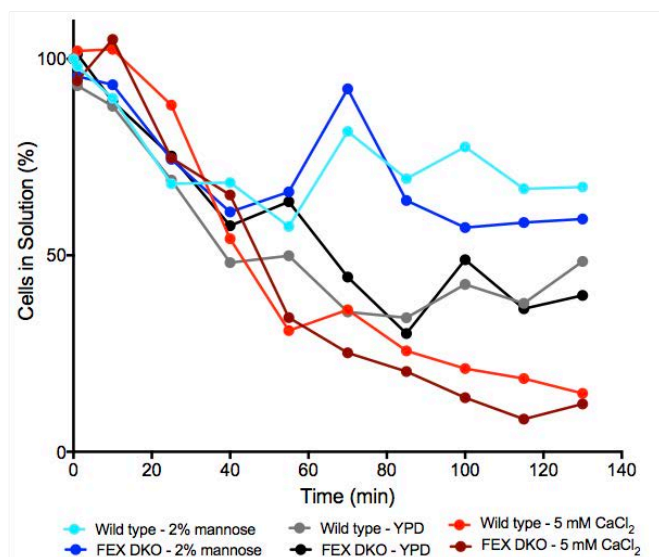


**Figure 9: Yeast growth after initial sedimentation.** Optical density of yeast in 1 mL YPD over 24 hours. Yeast are either constantly shaking at 30°C, or are kept in 30°C without shaking for the indicated time (denoted as “lag” time). The optical density is recorded after the lag steps are completed, for 24 hours with shaking. (A) Two fluoride-hardened mutant strains, (B) FEX DKO or (C) wild type yeast are exposed to their IC<sub>50</sub> and IC<sub>99</sub> values of fluoride exposure in 2% glucose. (D) FEX DKO yeast are exposed to their IC<sub>50</sub> and IC<sub>99</sub> values of fluoride exposure in 2% glucose, plus 2% mannose.

This rescue was most prominent at the minimum inhibitory concentration, at which cells can no longer grow in fluoride. We also tested for the consequence of the lag step in fluoride resistance in FEX DKO cells. We found a similar, although smaller degree of growth rescue at high fluoride (Fig. 9B). Yeast that were placed in solution without shaking entered the log and plateau phases at their associating time delays; for example, cells grown with no fluoride and a four-hour delay reached those phases four hours earlier than control. This indicates that the yeast were growing at approximately the same rate without shaking. FEX DKO cells had decreased growth at 50  $\mu\text{M}$  fluoride when the lag step was introduced, but had increased growth at 100  $\mu\text{M}$  fluoride. The degree of rescue was enhanced with an increase in lag time, up to 12 hours. These results suggest that sedimentation in weakly flocculating yeast results in fluoride resistance, but only around the minimum inhibitory concentration. We also tested for this effect in wild type yeast (Fig. 9C). In wild type yeast, there was no enhanced fluoride resistance upon sedimentation, indicating that this phenotype is only attributed to resistance against low levels of fluoride.

There are many possible mechanisms to explain why sedimentation rescues from high fluoride exposure. Fluoride triggers predominantly intracellular toxicity. Clustering cells would have reduced total surface area compared with suspended cells, and therefore less available area for fluoride entry. Furthermore, as this effect is only seen in the weakly flocculating strains, sedimentation may help drive a more flocculant state. Cell adhesion and flocculation has been reported by several labs to result in enhanced survival to aging, oxidative stress, and nutrient starvation. As fluoride toxicity leads to metabolic arrest and oxidative stress, flocculation would be a logical resistance mechanism.

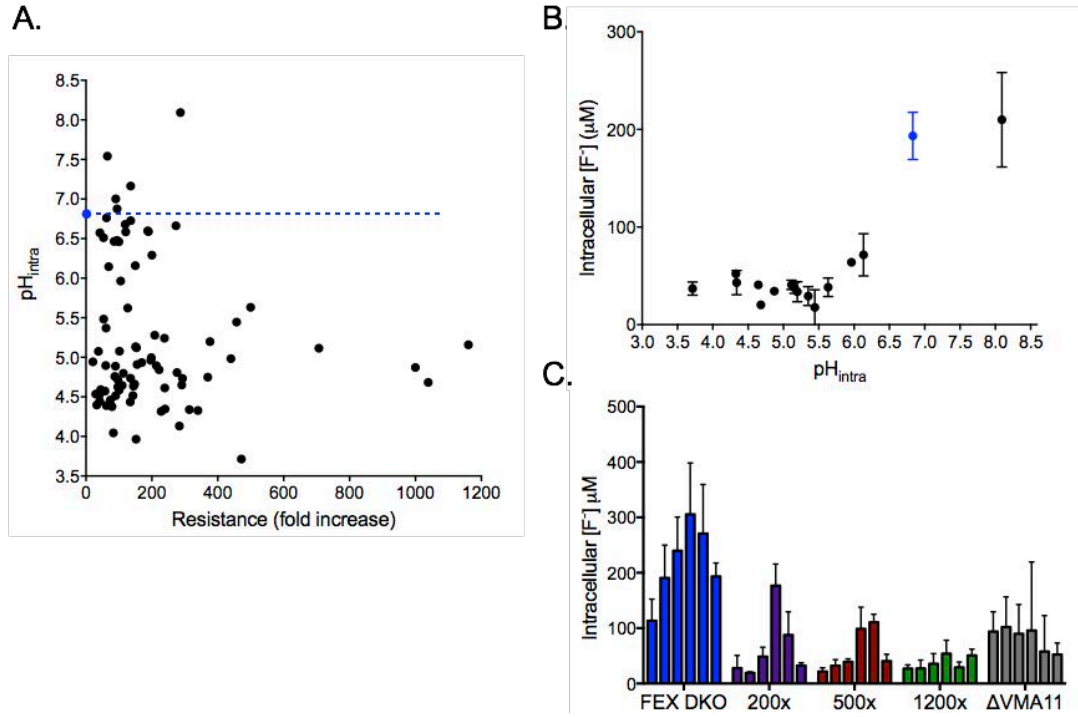
Of note, the FEX DKO and wildtype yeast in our lab appear to have weak, but present flocculation abilities. The wild type strain is BY4741, which is considered non-flocculating. BY4741 contains the genes essential for flocculation, but these are believed to be transcriptionally and epigenetically silenced. The flocculation number for FEX DKO at one minute is less than 10, which is considered very weak (Fig. 7C). Nonetheless, after approximately 60 minutes in solution, the rate by which both FEX DKO and wild type yeast sediment out of solution is dependent on conditions that enhance or disrupt flocculation (Fig. 10). Furthermore, the addition of mannose to solution reverses the rescue from fluoride toxicity with a lag step (Fig. 9D). Collectively, this suggests that BY4741 yeast have flocculation abilities, but at low levels that are enhanced by the opportunity to adhere to a surface prior to shaking.



**Figure 10: Flocculation rate in wild type and FEX DKO cells.** The percent of cells in solution at O.D. 5 are monitored for two hours in either 2 mL of (black/grey) YPD, (blue) 2% mannose, which disrupts flocculation, or (red) 5 mM calcium chloride, which promotes flocculation.

***Intracellular acidification prevents fluoride accumulation*** - The fluoride-hardened mutants displayed trends of metabolic dormancy and slowed growth. While under normal conditions cytoplasmic pH is tightly controlled, under stress conditions yeast can achieve dormancy by an influx of protons into their cytoplasm (Munder *et al.* 2016). Cellular acidification has a direct impact on the membrane potential, and consequent function of organelles. Given that the fluoride-hardened mutants had slowed growth, lowered metabolism, and disrupted membrane potentials in vacuoles and mitochondria, we hypothesized that the cells had shifted their intracellular pH. We tested for an altered cytoplasmic pH using a pH-sensitive dye (Fig. 11A). While the trend was not completely linear to the fold increase to fluoride resistance, in general mutants had reduced intracellular pH. Mutants with the highest resistance to fluoride averaged around pH 5.0, including those without ATPase mutations. Intracellular pH between 4-5 has been reported in yeast cells with altered metabolism. As the majority of mutants have mutations in metabolic genes, this seems the most likely contributor. Many other reported mutations may contribute to the intracellular acidification, particularly the gene CSS1. CSS1 had 107 total mutations across strains, including in three of the four mutants with  $\geq 700$ -

fold increase fluoride resistance. The function of CSS1 is unknown, but the gene is reported to be induced in cells with low or high pH. Most likely, a combination of mutations are responsible for the change in intracellular pH. Regardless of the mutation responsible for this phenotype, the majority of fluoride-resistant strains appear to be more acidic than FEX DKO.



**Figure 11: Intracellular pH and resulting fluoride accumulation in fluoride resistant mutants.** (A) Graph of increased fold-resistance to fluoride (as determined by liquid growth assays) and intracellular pH during log phase for each mutant. The blue represents the measured value for FEX DKO, and the dashed line is the theoretical intracellular pH of mutants had they not been altered. (B) 15 mutants (1 alkaline, 14 acidic) and their intracellular pH versus concentration of intracellular fluoride after 24 hours exposure to 50  $\mu M$  NaF. In blue is FEX DKO exposed to 50  $\mu M$  NaF for 24 hours. (C) Intracellular fluoride concentration over time. The clustered columns represent 2, 4, 6, 12, 18, and 24 hours of exposure to 50  $\mu M$  NaF for each strain. The strains are FEX DKO, 200x, 500x, and 1200x fluoride-hardened mutants, and FEX DKO with the V-ATPase subunit VMA11 deleted.

Five fluoride-hardened mutants had higher intracellular pH compared to FEX DKO yeast. Conserved mutations in these alkaline mutants showed a large degree of overlap with other, more acidic mutants (Table 4). The most commonly mutated genes included IPP1, FLO proteins, tRNAs, telomeres, DAN4, and ERR3. These genes were also commonly mutated in acidic mutants. As such, these alkaline mutants appear to follow the same general mechanisms of resistance to fluoride as acidic mutants. Given that the majority of higher-fold fluoride resistant mutants show intracellular acidification, acidification appears to be an important, independent mechanism for resistance to fluoride.

	Gene	Name	Total Mutations
1	YAL063C	FLO9	19
2	YMR317W	Unknown	12
3	YAR050W	FLO1	7
4	YMR319C	FET4	7
5	YBR011C	IPP1	6
6	YIL169C	CSS1	6
7	YBL101C	ECM21	5
8	YJR151C	DAN4	5
9	YMR316W	DIA1	4
10	YMR318C	ADH6	4
11	YMR323W	ERR3	4
12	tD(GUC)N	tRNA-Asp	3
13	tF(GAA)B	tRNA-Phe	3
14	YDL245C	HXT15	3
15	YER052C	HOM3	3
16	YER053C	PIC2	3
17	YGR040W	KSS1	3
18	YGR055W	MUP1	3
19	YIL152W	VPR1	3
20	YIL154C	IMP2	3
21	YIR017C	MET28	3
22	YIR019C	FLO11	3
23	YJR145C	RPS4a	3
24	YJR146W	Unknown	3
25	YLL005C	SPO75	3
26	YMR322C	SNO4	3
27	TEL08L	TEL08L	2
28	TEL13R	TEL13R	2
29	tL(UAA)B1	tRNA-Leu	2
30	tM(CAU)O1	IMT1	2

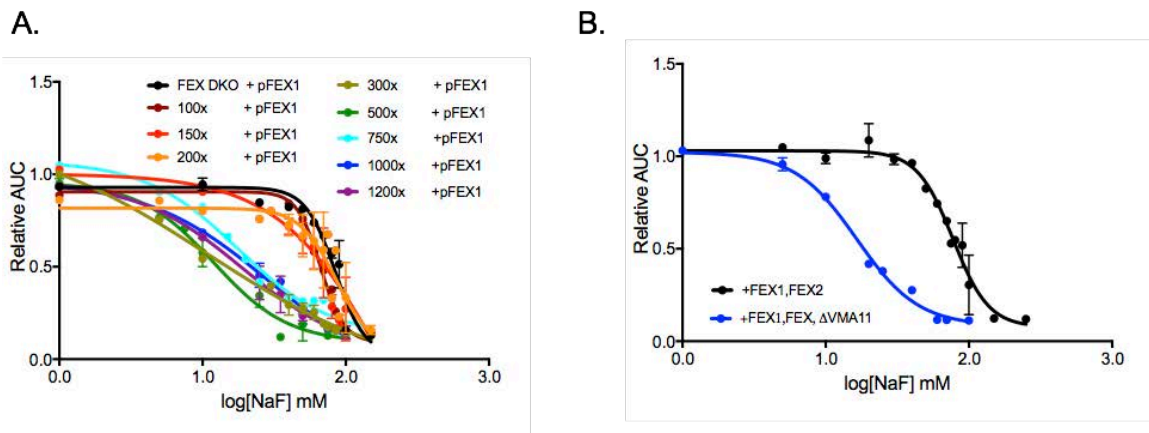
**Table 4: Top 50 conserved mutations across the five fluoride-resistant strains with alkaline intracellular pH.**

Hydrogen distribution affects fluoride's localization. Organisms are most sensitive to fluoride under acidic environments, as protonated fluoride can readily permeate cell membranes (Ji *et al.* 2014). Consequently, an acidic environment results in a gradient that shifts fluoride into cells. Reversing that gradient should then reverse the directionality of fluoride into leaving cells. To test this hypothesis, we monitored the accumulation of intracellular fluoride over 24 hours in 15 mutants (Fig. 11B). The mutant with an alkaline pH had no significant difference in intracellular fluoride concentration. Conversely, the acidic mutants all showed significantly reduced intracellular fluoride. There was approximately a 3-fold drop in intracellular fluoride in mutants at pH<sub>intra</sub> 6.0, and an additional 2-fold drop in intracellular fluoride in more acidic mutants.

To determine whether the reduced intracellular fluoride accumulation was consistent over time, we monitored fluoride accumulation at 2, 4, 6, 12, 18, and 24 hours for three mutants (Fig. 11C). These mutants consistently showed lower intracellular fluoride over time compared to FEX DKO. We then deleted VMA11

from FEX DKO to monitor its effect on fluoride accumulation. VMA11 is an essential subunit of V-ATPase, and disruption of V-ATPase through this deletion resulted in yeast intracellular pH of 5.9 (data not shown). Over 24 hours, the VMA11 knockout had similarly reduced intracellular fluoride as the hardened mutants, suggesting that intracellular acidification was directly responsible for fluoride excretion.

FEX is the only known yeast protein to transport fluoride. We transformed mutants with a high-copy plasmid containing FEX1 to determine whether FEX1 expression led to increased intracellular fluoride removal. Surprisingly, mutants expressing FEX1 were sensitized to fluoride compared with wild type (Fig. 12). Yeast sensitization correlated with intracellular pH, whereby the most sensitized mutants were also the most acidic. However, rather than a linear decrease, only strains with more than 300-fold increased fluoride resistance demonstrated a 3 to 7-fold loss in fluoride resistance with FEX1 expression. As indicated by <sup>31</sup>P NMR, this range of mutants are those that have lost vacuolar function (Fig. 6A). Together, the indicated trend suggests that FEX activity is somehow dependent on intracellular pH and vacuolar function.



**Figure 12: Effect of FEX1 and pH on fluoride resistance.** Fluoride resistance was monitored using liquid growth assays for (A) FEX DKO or mutant strains transformed with pRS416-FEX1 or (B) wildtype BY4741 strain (+FEX1, FEX2), and wildtype with the gene VMA11 deleted. Vma11p is an essential component of V-ATPase, and nonfunctional V-ATPase eliminates the pH gradient between the cytoplasm and vacuole, resulting in intracellular acidification.

## Summary

Here we report the generation of 81 *S. cerevisiae* strains that with mutations that lead to resistance to fluoride toxicity. These mutations are independent of the activity of a fluoride channel. Overall, we found that these mutants had slower growth, decreased metabolism, and increased cytoplasmic acidity.

Among these 81 fluoride-hardened strains, a total of one-fourth of yeast genes were altered. Given that, there are many possible genes and pathways that can be contributing to fluoride resistance. Our proposed mechanism of resistance through nutrient sharing and intracellular acidification is based on prominent overall trends that correlate with degree of resistance. Among these, a strong correlator to fluoride resistance was slowed growth. Slowed growth in yeast confers rescue from general stress through an unknown mechanism. One hypothesis is that slow growing yeast are better able to down-regulate essential genes that would otherwise be targeted by stressor. Slowed growth is triggered by many causes, including lowered metabolism, flocculation, and increased acidification.

Fluoride-hardened yeast had lowered metabolic rates and switched to a nutrient-sharing phenotype reminiscent of flocculation. It is well established that prolonged fluoride exposure leads to metabolic arrest, leading many in the field of fluoride toxicity to believe that glycolysis or mitochondrial function are the principle targets of fluoride. Previously, our lab reported that yeast lacking a fluoride channel undergo nutrient starvation when exposed to fluoride (Johnston and Strobel 2019). Nutrient sharing is an induced process in flocculating yeast during nutrient starvation conditions. Consequently, mimicking the nutrient sharing conditions and reducing metabolism in fluoride-hardened mutants promoted survival during fluoride exposure.

We also found that all yeast with more than 300-fold increased resistance to fluoride had acidified cytoplasm. Fluoride is unique to the halide family in that it has a positive pKa, allowing it to exist as HF in acidic environments and permeate cells. The lowered cytoplasmic pH of fluoride hardened yeast allowed for the excretion of intracellular fluoride as HF without the need of a protein channel. To our knowledge, this is the first reporting of a protein-free adaptation for removing an intracellular toxicant.



## Chapter 4: Genome-wide identification of genes involved in general acid stress and fluoride toxicity in *Saccharomyces cerevisiae*

*This section represents a manuscript submitted for publication. The manuscript hinges on a yeast knockout screen across five different types of acids. The library screen was tested with the first acid by Katie Smith and Patricia Gordon. The remaining four acids were screened by myself and Sunitha Nallur. The figures, analysis, and follow-up experiments were conducted by myself. The manuscript was prepared by myself and Scott A. Strobel.*

### **Abstract**

Hydrofluoric acid elicits cell cycle arrest through a mechanism long been presumed to be linked with the high affinity of fluoride to metals. However, our lab has recently found that the acid stress from fluoride exposure is sufficient to elicit many of the hallmark phenotypes of fluoride toxicity. Here we report the systematic screening of genes involved in fluoride resistance and general acid resistance using a genome deletion library in *Saccharomyces cerevisiae*. We used a variety of acids – 2,4-dinitrophenol, FCCP, hydrochloric acid, and sulfuric acid – none of which has a high metal affinity. We found that pathways involved in endocytosis, vesicle trafficking, pH maintenance, and vacuolar function are of particular importance to fluoride tolerance. The majority of genes conferring resistance to fluoride stress also enhanced resistance to general acid toxicity. Genes whose expression regulate Golgi-mediated vesicle transport were specific to fluoride resistance, and may be linked with fluoride-metal interactions. These results support the notion that acidity is an important and underappreciated principle underlying the mechanisms of fluoride toxicity.

### **Introduction**

Responsiveness to acid stress is an essential adaptation for microorganisms. Unlike multicellular organisms in which internal, sensitive tissues are protected from toxicants, microbes are directly exposed to their environment. As a result, a microbe's survival is dependent on the evolution of proteins and pathways capable of mitigating any potential stressors.

Eukaryotic microbes, such as fungi, contain organelles for the compartmentalization and specialized functions in combating acid stress. The intracellular pH of the cytoplasm and organelles are tightly controlled

by H<sup>+</sup>-ATPases, particularly Pma1p and V-ATPases (Kane 2016). This regulation is critical for maintaining the function of intracellular proteins, many of which are sensitive to pH changes. Of particular sensitivity are transmembrane and metabolic enzymes, such as phosphate transporters and phosphofructokinase (Hardewig *et al.* 1991; Ding *et al.* 2013). During acidosis, fungi lower metabolism and protein synthesis, and increase the production of saturated lipids and ergosterol to adjust membrane fluidity (Sousa *et al.* 2012; Brandao *et al.* 2014; Guo and Olsson 2016; Godinho *et al.* 2018; Palma *et al.* 2018). Cells also undergo remodeling of their plasma membranes and cytoskeletal components (Mollapour *et al.* 2006; Guo *et al.* 2018). While these are general response mechanisms to acidosis, different acids often elicit unique stress phenotypes based on their given properties.

Fungi routinely encounter acids in natural environments. Strongly acidic environments feature charged ions that cannot readily cross biological membranes without protein transporters. Consequently, exposure of fungi to highly acidic extracellular environments disrupts the electrochemical potential and function of the fungal plasma membrane (Carmelo *et al.* 1996; Russell and Gould 2003; Johnston and Strobel 2019). In contrast, exposure of fungi to weak acids can result in intracellular acidification (Mira *et al.* 2010). Weak acids, such as carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) and 2,4-dinitrophenol (DNP), act as protonophores, shuttling protons across biological membranes (Loomis and Lipmann 1948; Benz and McLaughlin 1983; Kenwood *et al.* 2014; Geisler 2019). Prolonged exposure to FCCP and 2,4-DNP uncouples oxidative phosphorylation of the mitochondria (Loomis and Lipmann 1948; Brennan *et al.* 2006). While this was presumed to be due to proton shuttling across the mitochondrial membrane and the consequent disruption of mitochondrial membrane potential, it was recently found that the phenotype was dependent on the acidified cytoplasm (Berezhnov *et al.* 2016). Given that different acids cause distinct toxicity phenotypes, studying the cellular response to an acid gives insight into its mechanism of toxicity.

Hydrofluoric acid is a weak acid with a pK<sub>a</sub> of 3.2 and is highly abundant in the environment. Fluoride inhibits metabolism, acidifies the cytoplasm, and elicits oxidative stress in cells (Feig *et al.* 1971; Kawase and Suzuki 1989; Hamilton 1990; Barbier *et al.* 2010). The mechanism behind these phenotypes has been assumed to be linked with the high affinity of fluoride for metals. *In vitro*, fluoride has been demonstrated to sequester the metals from the active site of essential metalloproteins, but only at millimolar fluoride concentration (Adamek *et al.* 2005). Our lab recently discovered that *Saccharomyces cerevisiae* lacking

fluoride exporters undergoes cell cycle arrest at 50  $\mu$ M NaF, well below the concentration required for any known metalloprotein inhibition (Li *et al.* 2013). We consequently found that fluoride induced cytoplasmic acidosis in yeast, resulting in a disruption of membrane potential and nutrient uptake (Johnston and Strobel 2019). From this, the question becomes: What aspects of fluoride toxicity are the direct consequence of acid stress?

Yeast genomic libraries serve as powerful tools for high-throughput screens. Genetic knockout libraries, encapsulating the deletion of each nonessential gene in the yeast genome, can be used to identify genes essential for a particular function (Giaever and Nislow 2014). In the context of acid tolerance, deletion of any gene involved in crucial resistance pathways will result in sensitivity to that acid. By comparing the genetic resistance pathways for acids of known toxicity with fluoride we can identify both overlapping essential genes, and genes that are specific to fluoride. Here we report the analysis of genes important for fluoride resistance, and compare them to genes involved in reducing extracellular and intracellular acidosis. We selected acids that do not chelate strongly to metals to distinguish acid stress effects from inhibition of metalloproteins. We report that several genes linked to Golgi-vesicle transport are unique to fluoride toxicity. Nonetheless, there is significant overlap in essential genes between fluoride and general acid stress.

## **Materials and Methods**

2.1 Media, strains, and knockout library: The yeast strain used in this study was BY4741. Yeast were typically grown in YPD buffer, containing 1 g yeast peptone (Becton, Dickinson and Co.), 0.5 g yeast extract (Becton, Dickinson and Co.), 50  $\mu$ L of 1% adenine (Sigma), and 2.5 mL of 40% glucose (Sigma) per 50 mL total volume in water. YPD-agar plates consist of YPD, plus an additional 1 gr agar per 50 mL solution (Becton, Dickinson and Co.). Sodium fluoride (Sigma Aldrich), HCl (Sigma Aldrich), H<sub>2</sub>SO<sub>4</sub> (Sigma Aldrich), 2,4-DNP (Sigma Aldrich), and FCCP (Sigma Aldrich) were used in the reported experiments. The knockout library is commercially available (Dharmacon). Knockout strains were inoculated overnight in YPD, then incubated on YPD agar +/- acid at starting O.D. 0.67 at 30°C until fully grown, typically 48 hours. Some genetic deletions resulted in slower growth, and required addition time.

2.2 Liquid growth assay and serial dilutions: Liquid growth assays were conducted over 24-hr intervals, as described in Li *et al.* 2013. Agar plates used in serial dilutions were placed in the 30°C incubator for 48 hr before imaging. Serial dilutions were prepared using the standard protocol.

2.3 Determining pH<sub>intra</sub> and pH<sub>extra</sub>: Cells were grown in 2 mL YPD +/- acid at starting O.D. 0.1. After four hours of growth at 30°C, the cells were harvested by spinning and resuspending in PBS. The pH of the YPD buffer (pH<sub>extra</sub>) was measured using a pH probe. The pH of the cells (pH<sub>intra</sub>) was measured using 5(6)-carboxyfluorescein diacetate (CFDA) dye under its standard protocol. The pH was determined by comparing fluorescence of each cell to a standard curve of yeast permeabilized using 70% ethanol, then resuspended in PBS with pH ranging from 3.5-7.5.

2.4 Bioinformatics analysis of genetic knockout screen: Genes were grouped by pathway based on their descriptions on the *Saccharomyces* Genome Database (yeastgenome.org). Network clusters were generated using the String Enrichment app, MCode, and ClueGO on Cytoscape (version 3.7.2). Enrichment ratios and p-values were generated using WebGestalt (<http://www.webgestalt.org>). Values were transferred to Prism Software for graphing.

2.5 Monitoring ROS production: Yeast (wild type or  $\Delta$ VMA11) were grown to log phase, and then added at O.D. 0.5 to 1.5 mL YPD +/- acids. The acids were at the IC<sub>50</sub> concentration: 75 mM NaF, 20  $\mu$ M FCCP, 0.3 mM 2,4-DNP, 120 mM HCl and 30 mM H<sub>2</sub>SO<sub>4</sub>. Cells were shaken at 30°C for 6 hrs, and then spun and washed three times in PBS. Yeast were resuspended in 100  $\mu$ L PBS and 10  $\mu$ M dihydroethidium dye, shaken, and incubated in the dark for 5 min. Relative fluorescence units were measured using a plate reader,  $\lambda_{\text{excitation}} = 480$  nm and  $\lambda_{\text{emission}} = 580$  nm. Fold change was calculated by comparing fluorescence of each sample to the average fluorescence of wild type yeast grown in YPD for 6 hr, adjusted by cell count.

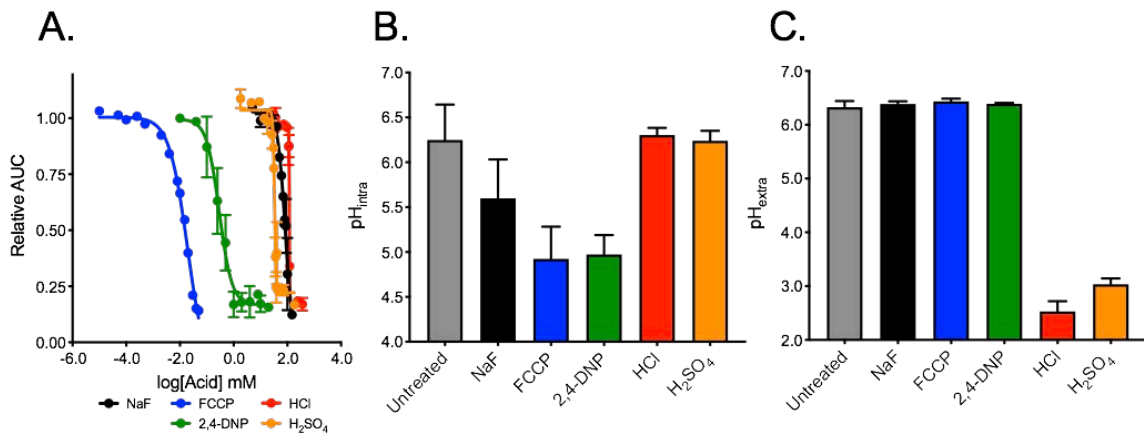
2.6 Assessing glucose uptake: Yeast were grown for four hr at 30°C starting at O.D. 0.5 in 2 mL YPD +/- acids. The acids were tested at the IC<sub>50</sub> for each: 75 mM NaF, 20  $\mu$ M FCCP, 0.3 mM 2,4-DNP, 120 mM HCl and 30 mM H<sub>2</sub>SO<sub>4</sub>. Cells were then washed three times in PBS. Yeast were resuspended in 100  $\mu$ L PBS with 1  $\mu$ L 2-NBDG, a fluorescent glucose mimic. Yeast were placed into a water bath at 30°C for one hr, washed three times in PBS, and resuspended into 100  $\mu$ L PBS. Fluorescence was analyzed with a plate reader at  $\lambda_{\text{excitation}} = 494$  nm and  $\lambda_{\text{emission}} = 521$  nm, and adjusted by cell count.

2.7 Monitoring intracellular polyphosphate with <sup>31</sup>P NMR: *In vivo* NMR was conducted with yeast grown to log phase, as outlined in Johnston and Strobel 2019.

## Results

**Non-essential genetic deletion screen for enhanced acid sensitivity** - We set out to distinguish what aspects of fluoride toxicity are most likely associated with acid stress rather than metalloprotein inhibition. In order to establish this distinction, we also introduced cells to HCl, H<sub>2</sub>SO<sub>4</sub>, FCCP, or 2,4-DNP; each of which illicit acid stress but do not have a high affinity for metals.

We first determined the concentration range of each acid required to illicit growth arrest over 24 hours in wildtype yeast (Fig. 1A). The IC<sub>50</sub>'s ranged broadly between the acids, with the most potent being FCCP and 2,4-DNP (0.02 and 0.3 mM, respectively) and the least being sulfuric acid (30 mM), sodium fluoride (75 mM), and hydrochloric acid (120 mM).



**Figure 1: Toxicity of acids in yeast.** (A) The toxic range of each acid in inhibiting yeast growth is assessed over 24 hours by a liquid growth assay, with increasing concentrations of each acid. Yeast were then exposed to acids at their IC<sub>50</sub> concentrations for four hours, and the (B) intracellular and (C) extracellular pH of the yeast was established using pH-sensitive dye and a pH electrode, respectively.

The addition of weak acids FCCP, 2,4-DNP, and NaF causes intracellular acidification, but not extracellular acidification (Fig. 1B and 1C). These acids have positive pK<sub>a</sub>s of 6.2, 4.1, and 3.2, respectively. Consequently, a subset of each acid would be in its protonated form inside the cell and able to pass through lipid compartments. In contrast, the strong acids HCl and H<sub>2</sub>SO<sub>4</sub> with pK<sub>a</sub>s of -6.3 and -2.0, respectively, do not exist in nature in their protonated form, and only cause extracellular acidification. In this way, fluoride is the most like 2,4-DNP and FCCP. It does not significantly alter the pH of the outside media, and induces

intracellular acidification at 4 hours exposure. However, the degree of intracellular acidification for fluoride is not as great as 2,4-DNP or FCCP at their respective IC<sub>50</sub>'s.

A commercially available *S. cerevisiae* knockout library was used to identify genes that confer resistance to acid stress. This library consists of single deletions in 5,250 non-essential genes. We exposed all 5,250 yeast knockout strains to the lowest observed adverse effect level (LOAEL) and IC<sub>25</sub> concentrations of each acid on YPD agar plates and allowed the strains to grow to saturation. Across all five acids, a total of 4,908 genetic deletions caused no noticeable sensitivity to stress. 342 genetic deletions resulted in significant growth arrest under acid exposure (Table 1). 133 of genetic deletions resulted in sensitivity to fluoride, 132 genetic deletions resulted in sensitivity to H<sub>2</sub>SO<sub>4</sub>, while 161 total deletions resulted in sensitivity to HCl. Exposure to either of the weak acids FCCP or 2,4-DNP was sensitized by 204 or 148 genetic deletions, respectively.

**Table 1**

Name	ORF name	high FCCP	high DNP	high H <sub>2</sub> SO <sub>4</sub>	high HCl	high NaF
<b>Mitochondria</b>						
AEP1	YMR064W		x			
AFG3	YER017C	x	x	x	x	x
ATP7	YKL016C	x		x		
ATP17	YDR377W	x				
ATP22	YDR350C	x				
ATP25	YMR098C		x			
CBP2	YHL038C	x			x	
CCM1	YGR150C	x				
CEM1	YER061C	x				
COQ6	YGR255C	x	x			
COQ8	YGL119W	x	x			
COQ10	YOL008W	x				
COX10	YPL172C	x		x	x	
COX11	YPL132W	x				
COX18	YGR062C	x		x		
COX20	YDR231C					x
COX23	YHR116W	x		x		
CYC3	YAL039C	x		x	x	x
CYT2	YKL087C	x		x		

GEM1	YAL048C	x	x	x	x	x
GEP4	YHR100C	x	x			
GGC1	YDL198C	x	x		x	x
IBA57	YJR122W	x				
IMG1	YCR046C	x	x			
IMG2	YCR071C	x				
IMP2	YMR035W	x		x		
ISA2	YPR067W	x	x			
LIP2	YLR239C	x				
MBA1	YBR185C				x	
MDJ1	YFL016C		x			
MEF1	YLR069C	x	x			
MRF1	YGL143C		x			
MRP1	YDR347W	x	x		x	
MRP7	YNL005C	x	x			
MRP17	YKL003C	x				
MRP20	YDR405W	x				
MRP21	YBL090W	x	x	x	x	x
MRP51	YPL118W	x			x	
MRPL4	YLR439W	x	x			
MRPL6	YHR147C	x				
MRPL7	YDR237W	x				x
MRPL8	YJL063C	x				
MRPL9	YGR220C	x				
MRPL13	YKR006C		x			
MRPL15	YLR312W-A	x				
MRPL16	YBL038W	x	x	x	x	x
MRPL17	YNL252C	x	x			
MRPL23	YOR150W		x			
MRPL27	YBR282W	x				
MRPL32	YCR003W	x	x			
MRPL36	YBR122C		x			
MRPL37	YBR268W		x			
MRPL40	YPL173W	x	x	x	x	
MRPS5	YBR251W	x	x			
MRPS35	YGR165W	x				
MRX14	YDR115W	x	x			
MSD1	YPL104W	x			x	
MSF1	YPR047W	x				
MSM1	YGR171C	x				

MSR1	YHR091C	x	x	x		
MSS116	YDR194C	x				
MSW1	YDR268W		x			
MSY1	YPL097W	x	x			
MTF1	YMR228W	x	x			x
MTG1	YMR097C		x			
NAM2	YLR382C	x	x			
OXA1	YER154W	x	x			
PCP1	YGR101W	x	x	x		
PET54	YGR222W	x	x			
PET100	YDR079W	x	x	x		
PET117	YER058W	x				
PET123	YOR158W		x			
QRI5	YLR204W	x		x		
QRI7	YDL104C	x	x			
RIP1	YEL024W			x	x	
RML2	YEL050C	x	x			
RPO41	YFL036W	x				
RSM7	YJR113C	x	x			
RSM18	YER050C	x				
RSM19	YNR037C	x	x			
RSM22	YKL155C		x			x
RSM24	YDR175C	x				
RSM27	YGR215W	x	x	x		x
SLM5	YCR024C					x
SLS1	YLR139C	x	x			
SSQ1	YLR369W		x			
SOV1	YMR066W		x			
TIM11	YDR322C-A		x			
YTA12	YMR089C	x	x			

**Metabolism/nutrient homeostasis**

ARO1	YDR127W		x	x		x
ARO2	YGL148W			x		x
CSF1	YLR087C	x		x		x
DUG2	YBR281C					x
GLY1	YEL046C		x			
HAP2	YGL237C	x	x			
HAP3	YBL021C			x		x
HAP5	YOR358W	x				



HOM6	YJR139C					X
ILV1	YER086W					X
KRE6	YPR159W					X
PFK1	YGR240C	x	x			X
PRS3	YHL011C	x		x		X X
PRS5	YOL061W					X
REG1	YDR028C					X
RIB1	YBL033C		x			
THR1	YHR025W					X
THR4	YCR053W					X
TPS1	YBR126C			x		
TRP3	YKL211C		x			
YGP1	YNL160W	x		x		X X
ZWF1	YNL241C			x		X X

**Growth**

BEM1	YBR200W	x	x	x		X X
BUD25	YER014C-A	x		x		X X
ILM1	YJR118C					X
IME4	YGL192W					X
PET130	YJL023C	x				
RHO4	YKR055W	x	x	x		X X
RSR1	YGR152C	x	x			X
SIN3	YOL004W	x		x		X X
SIT4	YDL047W			x		X

**DNA Regulation**

FYV6	YNL133C	x		x		X X
FZO1	YBR179C	x	x			
GEP5	YLR091W	x	x			
HTA1	YDR225W	x				X
HTB2	YBL002W		x			
HTL1	YCR020W-B	x	x	x		X X
IES2	YNL215W	x		x		X X
IES6	YEL044W					X
LCL3	YGL085W	x				
MEC3	YLR288C	x	x	x		
MGM101	YJR144W					
MHR1	YDR296W		x			
MIP1	YOR330C	x				

MSH1	YHR120W	x	x	x	x	x
RAD6	YGL058W	x				
SHU2	YDR078C	x	x	x		

**Stress Response**

ATG4	YNL223W			x	x	x
BCK1	YJL095W			x	x	
BOL2	YGL220W	x	x	x	x	
CNB1	YKL190W				x	
CRZ1	YNL027W					x
DBF2	YGR092W				x	
GON7	YJL184W	x		x	x	x
GPH1	YPR160W	x				
HOG1	YLR113W	x		x	x	
HRK1	YOR267C	x				
KCS1	YDR017C	x	x	x	x	x
KSS1	YGR040W	x				
PBS2	YJL128C				x	
PDE2	YOR360C	x		x	x	
RVS161	YCR009C		x			x
SNG1	YGR197C		x			
WAR1	YML076C	x				

**Protein Expression (Translation)**

ALG3	YBL082C				x	
ALG6	YOR002W				x	
ALG8	YOR067C				x	
BUD23	YCR047C			x	x	
CAX4	YGR036C	x	x	x	x	x
DCS1	YLR270W	x	x	x		
DHH1	YDL160C				x	
GIR2	YDR152W				x	
MEF2	YJL102W	x	x			
NCL1	YBL024W	x	x			x
OST3	YOR085W				x	
POP2	YNR052C	x	x	x	x	x
REF2	YDR195W	x	x		x	
RPL12B	YDR418W		x			
RPP1A	YDL081C				x	x
SAC3	YDR159W	x		x	x	

SED1	YDR077W				x	x	
SLM3	YDL033C	x	x		x	x	
TEF4	YKL081W			x			x

**Vesicle-Mediated Transport**

BRO1	YPL084W	x			x	x	x
CDC50	YCR094W	x			x	x	x
CHC1	YGL206C	x	x		x	x	x
CLC1	YGR167W	x	x		x	x	x
DID4	YKL002W	x			x	x	x
DRS2	YAL026C					x	
ERV14	YGL054C		x				
KES1	YPL145C						x
PEP3	YLR148W				x	x	x
PEP12	YOR036W	x	x		x	x	x
RVS167	YDR388W						x
SHE4	YOR035C	x			x	x	x
SNF7	YLR025W	x			x		x
SNF8	YPL002C	x			x	x	x
SRN2	YLR119W						x
STP22	YCL008C						x
SWF1	YDR126W				x	x	x
VAM3	YOR106W						x
VPS3	YDR495C				x	x	x
VPS4	YPR173C	x			x		x
VPS5	YOR069W				x	x	
VPS8	YAL002W						x
VPS9	YML097C						x
VPS16	YPL045W	x			x	x	x
VPS20	YMR077C	x			x	x	x
VPS21	YOR089C	x					x
VPS24	YKL041W						x
VPS25	YJR102C	x			x	x	x
VPS27	YNR006W	x			x	x	x
VPS28	YPL065W						x
VPS33	YLR396C		x				
VPS36	YLR417W						x

**Ion homeostasis**

AGP2	YBR132C	x	x		x	x	x
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AQR1	YNL065W	x		x	x	
ARN1	YHL040C	x				
CCC2	YDR270W					x
CCH1	YGR217W	x		x	x	
DAL5	YJR152W	x				x
ECM7	YLR443W				x	
GUP1	YGL084C	x		x	x	
JEN1	YKL217W	x				
MAC1	YMR021C	x	x			x
MCH5	YOR306C	x			x	x
MID1	YNL291C			x	x	
NHX1	YDR456W					x
NPR1	YNL183C					x
PDR12	YPL058C	x				x
PDR18	YNR070W			x	x	x
SKY1	YMR216C	x		x		x
TPO2	YGR138C	x		x		
TPO3	YPR156C	x				
TRK1	YJL129C				x	

**Protein Regulation**

ADD37	YMR184W	x	x			
APE2	YKL157W	x				
DAP2	YHR028C		x			
DOA4	YDR069C	x		x	x	x
DOC1	YGL240W		x			
KEX2	YNL238W	x	x		x	
PEX17	YNL214W					x

**Gene Expression (Transcription)**

AIM10	YER087W	x	x			
BUR2	YLR226W			x	x	
CCR4	YAL021C			x	x	x
CDC40	YDR364C	x	x	x	x	x
CYC8	YBR112C	x	x	x	x	x
HF11	YPL254W	x	x	x		
MOT2	YER068W	x		x	x	x
ROX3	YBL093C					x
RPB4	YJL140W	x				
RPH1	YER169W		x			

TAF14	YPL129W	x	x	x	x	x
TFB5	YDR079C-A	x	x	x		
TPD3	YAL016W	x	x	x	x	x
URE2	YNL229C	x				

### Lipid Metabolism

ARE2	YNR019W	x				
CHO1	YER026C	x	x	x	x	x
ELO2	YCR034W	x				
ERG2	YMR202W	x				x
ERG3	YLR056W		x		x	
ERG4	YGL012W		x			x
ERG24	YNL280C					x
LCB5	YLR260W	x		x		
LEM3	YNL323W		x			
MCT1	YOR221C	x				
TSC3	YBR058C-A	x	x	x	x	
VPS34	YLR240W			x	x	x

### Vacuole

PKR1	YMR123W	x	x	x	x	x
RRG1	YDR065W		x			
VAM7	YGL212W					x
VMA1	YDL185W		x		x	x
VMA2	YBR127C	x	x	x	x	x
VMA3	YEL027W	x	x	x	x	x
VMA5	YKL080W	x	x	x	x	x
VMA6	YLR447C	x	x	x	x	x
VMA7	YGR020C	x	x	x	x	x
VMA8	YEL051W		x	x	x	x
VMA9	YCL005W-A	x	x	x	x	x
VMA10	YHR039C-B	x	x	x	x	x
VMA11	YPL234C	x	x	x	x	x
VMA13	YPR036W	x	x	x	x	x
VMA16	YHR026W	x	x	x	x	x
VMA21	YGR105W	x	x	x	x	x
VMA22	YHR060W	x	x	x	x	x
VPH2	YKL119C	x	x	x	x	x
VPS1	YKR001C	x		x	x	x
VPS15	YBR097W			x	x	x

**Golgi**

ARF1	YDL192W						X
ANP1	YEL036C	x	x	x			X
COG1	YGL223C	x	x	x	x		X
GLO3	YER122C	x					
GOS1	YHL031C						X
GYP1	YOR070C		x				
HUR1	YGL168W	x			x	x	X
MNN10	YDR245W						X
OCH1	YGL038C	x	x	x	x		X
PMR1	YGL167C	x		x	x		X
SAC1	YKL212W						X
VPS52	YDR484W	x	x			x	X
VPS53	YJL029C	x		x		x	X
VPS54	YDR027C	x		x		x	X

**Endoplasmic Reticulum**

CSG2	YBR036C				x		x
DIE2	YGR227W						x
EUG1	YDR518W	x	x				
GET1	YGL020C	x	x				x
GET2	YER083C	x	x	x			x
PER1	YCR044C						x
SBH1	YER087C-B		x				

**Cell surface**

DCW1	YKL046C						x
GAS1	YMR307W	x			x		x
PMP3	YDR276C	x			x		x
SCW10	YMR305C				x		x
SLT2	YHR030C						x
SMI1	YGR229C						x

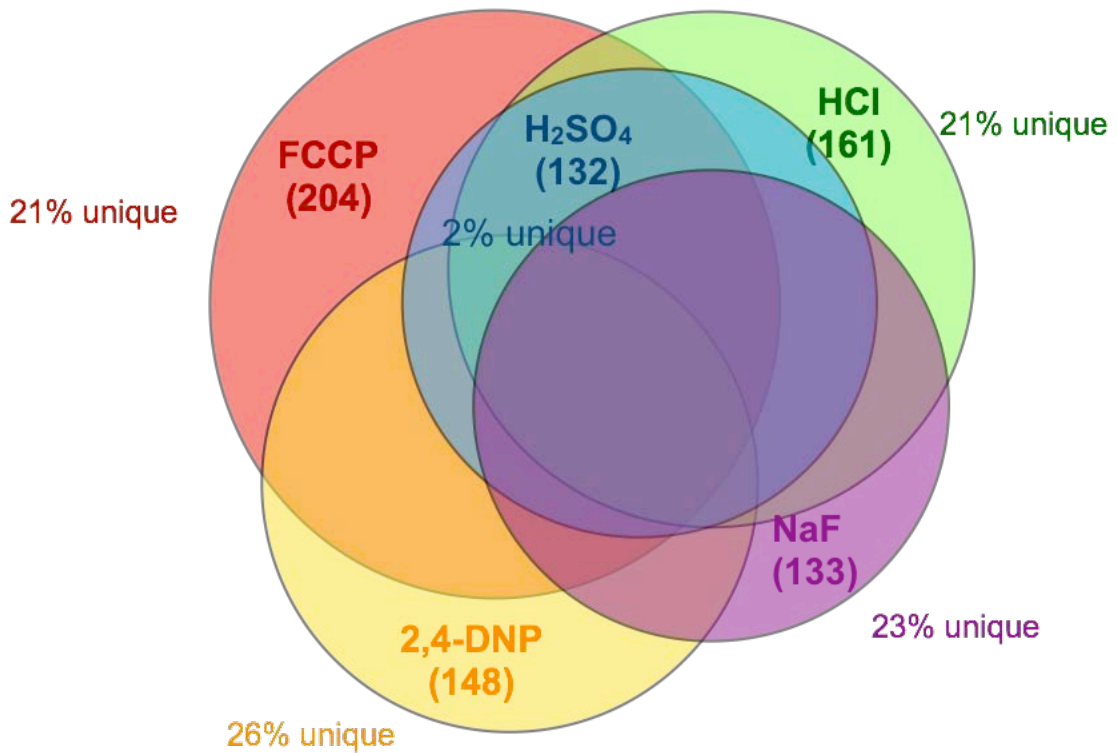
**Cytoskeleton**

CNM67	YNL225C		x				
HOF1	YMR032W						x
NIP100	YPL174C	x					x
SLA1	YBL007C	x			x		x
VRP1	YLR337C						x

**Unknown**

BRP1	YGL007W					x	
FAP1	YNL023C	x	x	x	x	x	x
FYV8	YGR196C					x	
RRG7	YOR305W		x	x	x		
RRG9	YNL213C	x	x	x	x		
SKG3	YLR187W					x	
VPS61	YDR136C						x
VPS63	YLR261C	x				x	
VPS69	YPR087W	x			x	x	x
YBL044W	YBL044W	x	x				
YBL094C	YBL094C					x	
YCL001W-B	YCL001W-B		x				
YDR008C	YDR008C		x				
YDR114C	YDR114C	x	x				
YDR169C-A	YDR169C-A		x				
YDR182W-A	YDR182W-A		x				
YDR455C	YDR455C						x
YGL007C-A	YGL007C-A		x	x	x		x
YGR035W-A	YGR035W-A	x	x	x	x		x
YGR219W	YGR219W		x				
YHL015W-A	YHL015W-A		x				
YKL096C-B	YKL096C-B	x	x			x	
YKL118W	YKL118W	x	x	x	x		x
YKL158W	YKL158W	x		x			
YMR320W	YMR320W					x	
YNL170W	YNL170W		x				
YNL184C	YNL184C					x	
YOR041C	YOR041C						x
YOR331C	YOR331C		x	x			x
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YPR099C	YPR099C	x		x	x		x
YPR172W	YPR172W					x	

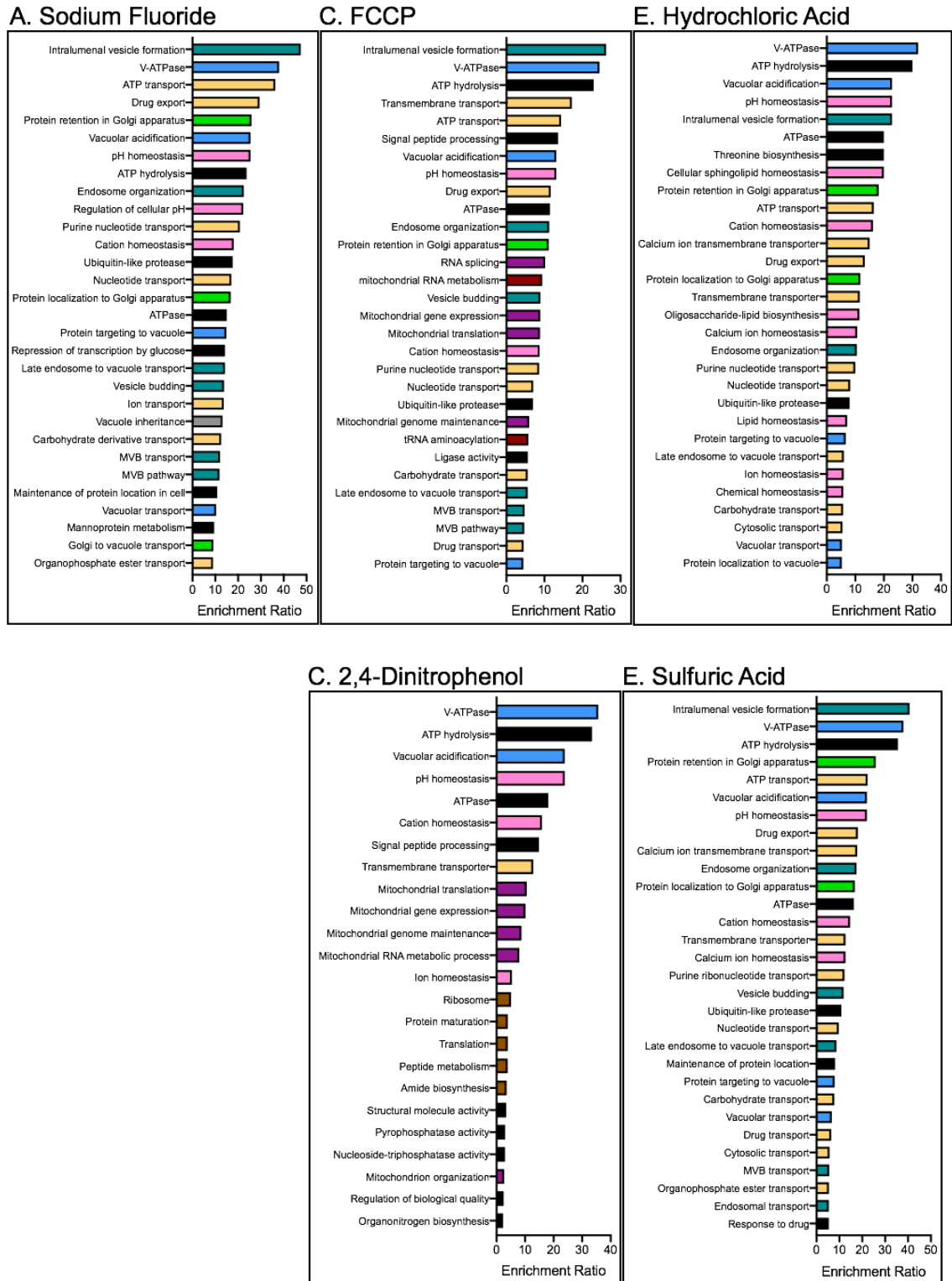
The overwhelming majority of genes caused sensitivity to more than one acid. Of the 132 genes conferring resistance to sulfuric acid, less than 2% were specific to sulfuric acid (Fig. 2). The other acids had roughly 20% of their gene list unique to that acid, including fluoride. This indicates that around 80% of genes conferring resistance to fluoride are part of general acid resistance.



**Figure 2: Venn Diagram of acid tolerance** to NaF, FCCP, 2,4-DNP, HCl, and H<sub>2</sub>SO<sub>4</sub> stress. Circles denote the relative size of the gene deletion list conferring sensitivity to that acid, and marked is the percent of the list unique to each acid.

We performed enrichment analysis using a series of programs in order to determine the biological pathways, organelles, and genes most important for acid resistance. First, we utilized the functional analysis tool WebGestalt to calculate the enrichment of biological processes and molecular functions within each dataset (Fig. 3).



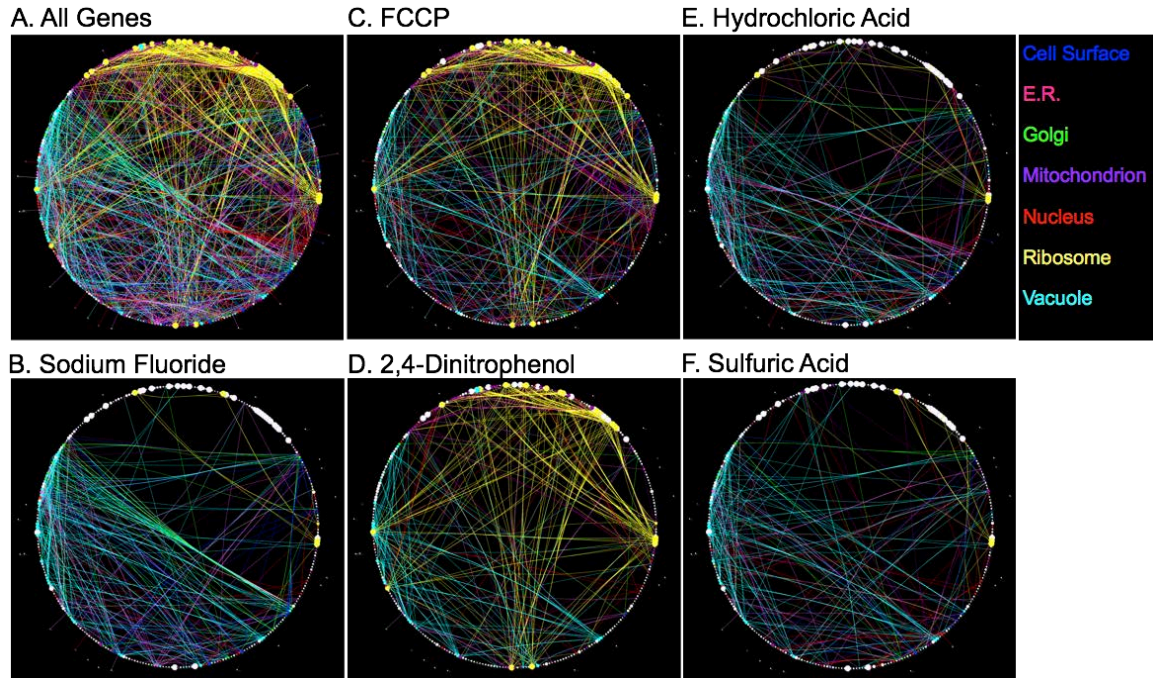


**Figure 3: Enrichment analysis for the biological processes and molecular functions of genes conferring resistance to (A) NaF, (B) FCCP, (C) 2,4-DNP, (D) HCl, and (E) H<sub>2</sub>SO<sub>4</sub> stress. Enrichment analysis was performed using WebGestalt, and redundant categories were eliminated. The top 30 most enriched pathways**

are reported above, having at least an enrichment ratio value of 2 and a p-value below 0.01. The colors corresponding to the columns are (green) – golgi, (pink) – ion homeostasis, (purple) – mitochondria, (red) – nucleus, (yellow) – protein transporters, (brown) – ribosomes, (blue) – vacuole, (turquoise) – vesicle-mediated transport, and (black) – other.

Datasets of sensitized genetic deletions for each of the five acids were enriched for those involved in vacuolar ATPase, pH maintenance, and vesicle formation. Genes involved in fluoride resistance were the most enriched for Golgi function. Genes involved in resistance to FCCP and 2,4-DNP were heavily enriched for mitochondrial function and DNA maintenance, as well as protein translation for 2,4-DNP resistance. Genes involved in resistance to strong acids HCl and H<sub>2</sub>SO<sub>4</sub> were heavily enriched in ion homeostasis and cell surface genes, including nutrient transporters.

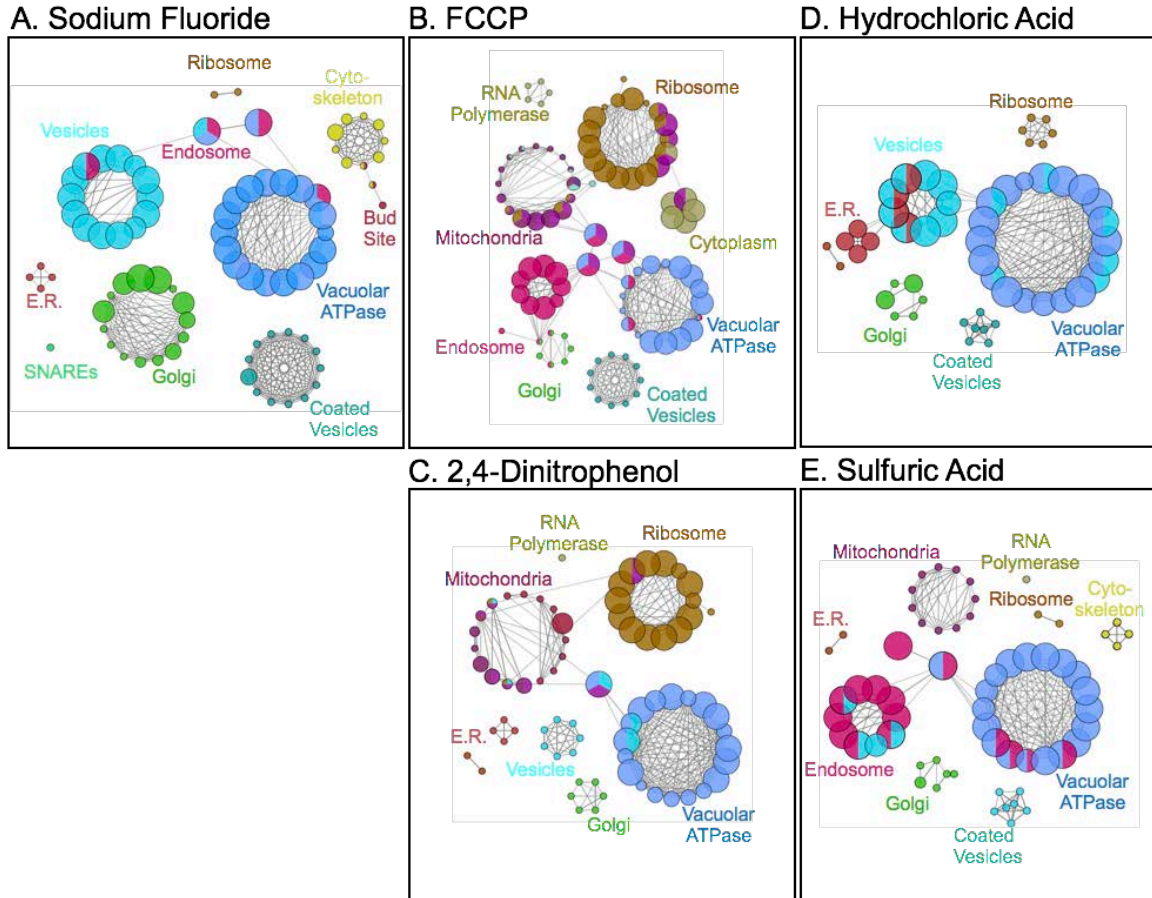
We then used the String Database to plot all 342 genes based on their corresponding protein-protein interaction networks (Fig. 4). Genes with a high degree of interaction were those associated with the ribosome and vacuole. Genes involved in fluoride tolerance were heavily enriched in vacuolar and Golgi interaction networks. Conversely, genes involved in weak acid tolerance were enriched in vacuolar and ribosomal networks, and those in strong acid tolerance were enriched in vacuolar and cell surface networks.



**Figure 4: Protein-protein interaction networks of genes whose deletion confer sensitivity to acid.**

Networks were plotted on Cytoscape using the String Database, and colored by their classification using the Yeast Genome Database Gene Ontology Slim Term Mapper. All 342 genes are plotted and colored in (A), and in later figures the color is removed except for genes that confer resistance to (B) NaF, (C) FCCP, (D) 2,4-DNP, (E) HCl, and (F) H<sub>2</sub>SO<sub>4</sub>.

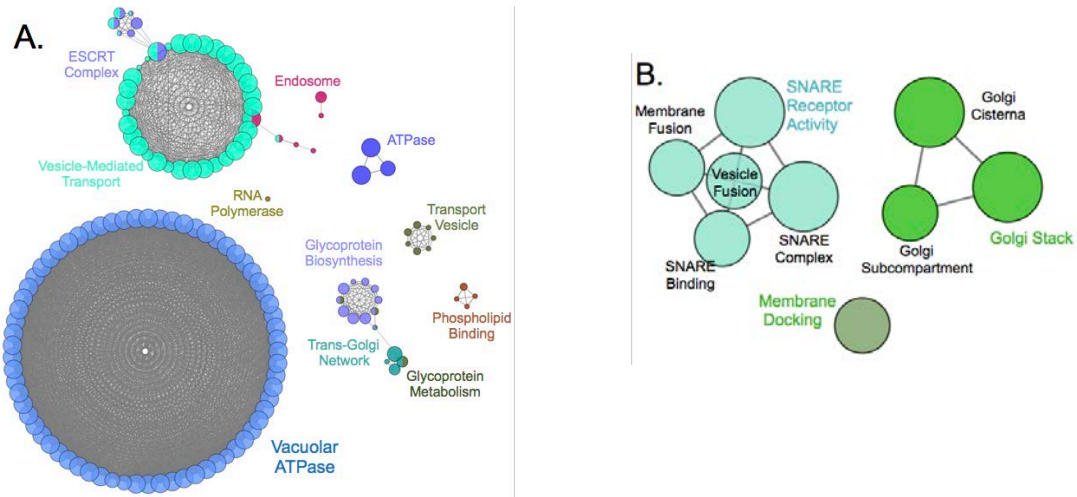
As a third method of bioanalysis, we utilized ClueGO to examine important cellular components of acid resistance (Fig. 5). Genes involved in mitochondrial and ribosomal function were enriched in response to weak acid stress caused by 2,4-DNP and FCCP exposure. 2,4-DNP had greater mitochondrial enrichment than FCCP, presumably both because FCCP had a larger dataset, and previous research has found that mitochondrial inactivation by FCCP is linked more to cytoplasm acidification than a direct interaction (Berezhnov *et al.* 2016). Genes involved in endosomal and vesicle-mediated transport processes were enriched in response to the strong acids HCl and H<sub>2</sub>SO<sub>4</sub>. The vacuolar ATPase genes – essential in pH maintenance - resulted in high sensitivity in response to exposure to any of the acids. Fluoride toxicity was selectively enhanced upon deletion of genes involved in cytoskeleton, vesicle-mediated transport, and Golgi function. Genes involved in Golgi, coated vesicles, and SNARE function were more enriched in response to fluoride stress than any other acid.



**Figure 5: Cellular components involved in resistance to (A) NaF, (B) FCCP, (C) 2,4-DNP, (D) HCl, and (E) H<sub>2</sub>SO<sub>4</sub> stress.** Images were composed using ClueGO software from pathways with  $pV \leq 0.050$ . Node size is proportional to the fraction of genes in that particular node.

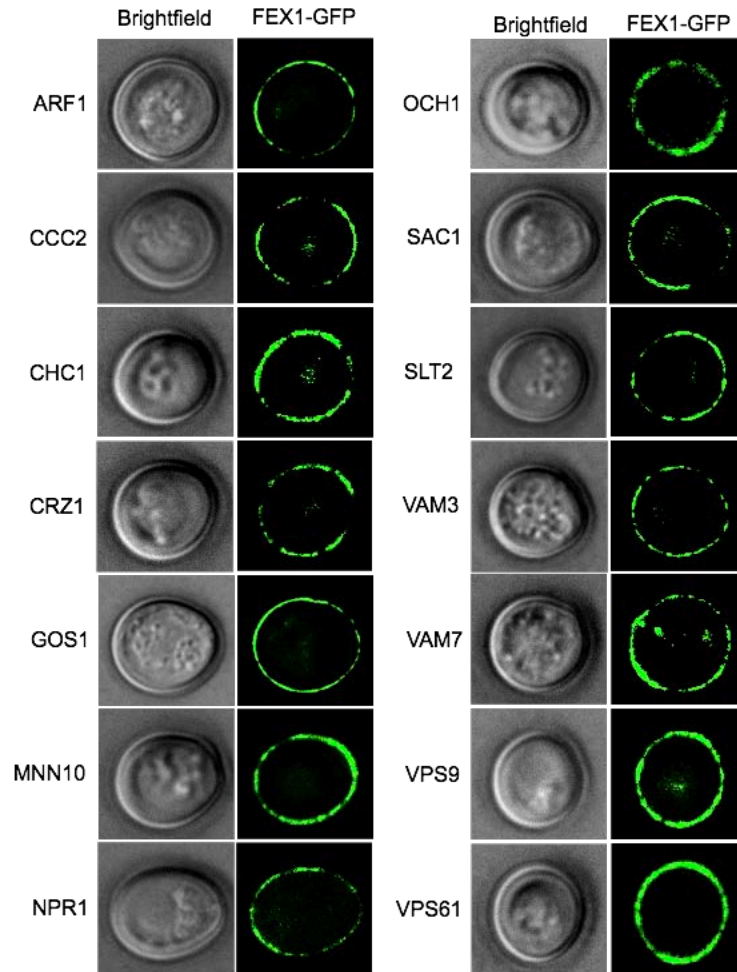
Of the 342 gene deletions that affected acid sensitivity, 39 deletions caused sensitivity to all six acids tested. Within these genes were significant enrichment for vacuolar ATPase and pH maintenance, vesicle-mediated transport, and glycoprotein production (Fig. 6A). 101 of 342 gene deletions affected both weak acids 2,4-DNP and FCCP, and 106 gene deletions affected both strong acids HCl and H<sub>2</sub>SO<sub>4</sub>. Of the 133 genetic deletions resulting in sensitivity to fluoride, 32 of those genes – ARF1, CCC2, COX20, CRZ1, ERG24, GOS1, HOF1, IES6, KES1, MNN10, NHX1, NPR1, PEX17, PRS5, ROX3, RPP1A, RVS167, SAC1, SLT2, SRN2, STP22, VAM3, VAM7, VPS8, VPS9, VPS24, VPS28, VPS36, VPS61, VRP1, YDR455C, and YOR041C – were unique to fluoride resistance. Gene ontology analysis of these genes

showed significant enrichment for Golgi function, ESCRT machinery, and SNARE receptor activity (Fig. 5B).



**Figure 6: Cellular components involved in resistance to (A) all acids, and (B) only NaF.** Images were composed using ClueGO software from pathways with  $pV \leq 0.050$ . Node size is proportional to the fraction of genes in that particular node.

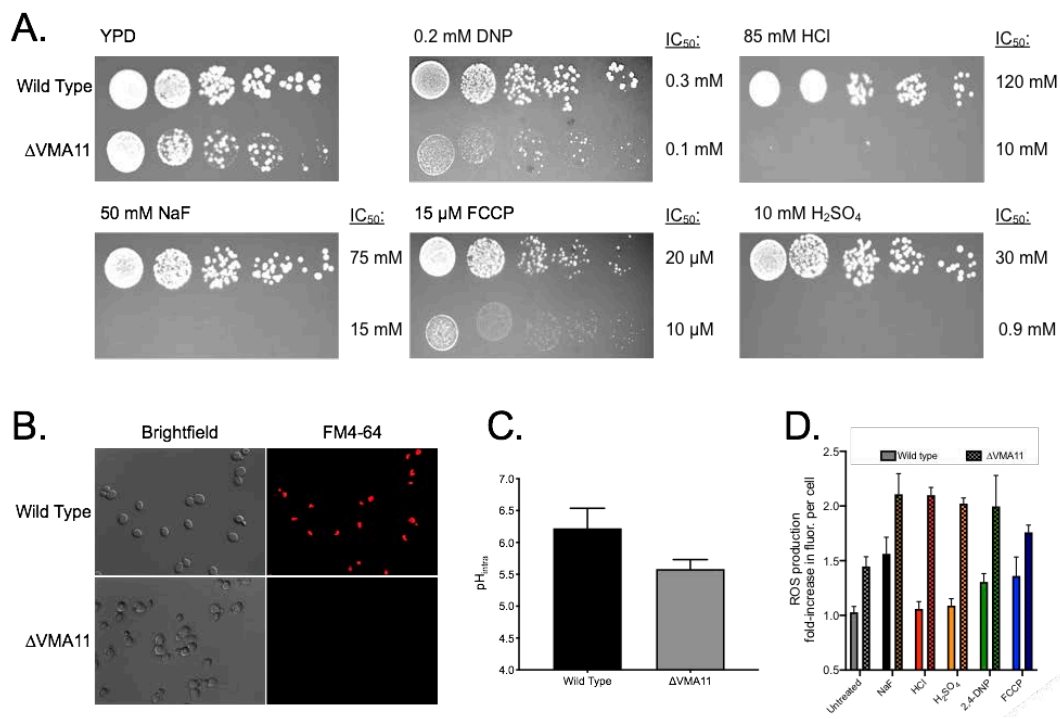
Fluoride resistance is heavily influenced by the expression of two fluoride transporters, FEX1 and FEX2. Our lab previously found that single deletions of fluoride transporters do not affect fluoride tolerance (Li *et al.* 2013). As expected, the single deletions did not appear in our screen to confer sensitivity to any acid. Conversely, deletion of both genes results in over 1000-fold increased sensitivity to fluoride. There is a possibility that Golgi function is necessary to successfully incorporate fluoride transporters to the cell membrane. To test this, we inserted plasmids containing GFP-FEX1 into 14 of the genetic deletions specific for fluoride resistance (Fig. 7).



**Figure 7: Images of GFP-tagged FEX** inserted into the denoted genetic knockout. These 14 genetic knockouts were screened for FEX1 insertion into the plasma membrane. All cells showed localization of GFP to the plasma membrane, suggesting that FEX1 has been successfully incorporated.

Microscopy analysis of the yeast demonstrated strong localization of the GFP signal to the plasma membrane, indicating the successful incorporation of FEX1. Furthermore, the genetic deletions specific to fluoride resulted in less than 10-fold increased fluoride sensitivity, as opposed to the 1000-fold observed by completely abolishing FEX expression. This is not necessarily unexpected, as genetic deletions that would completely abolish transmembrane protein incorporation would mostly likely be lethal. In all, it appears that the genes involved in Golgi function cause sensitivity unrelated to FEX localization.

***V-ATPase confers acid resistance through pH and ROS maintenance*** - Deletion of V-ATPase subunits resulted in significant sensitivity to all of the acids tested. This implicates that vacuolar ATPase is essential for general acid resistance. V-ATPase is a proton pump composed of 13 subunits. Deletion of 12 of the 13 subunits caused significant sensitivity to acid stress, and deletion of 10 of those subunits resulted in sensitivity to all six acids. Of these, deletion of VMA11 resulted in the greatest acid sensitivity (Fig 8A).  $\Delta$ VMA11 yeast had a 5-fold lower  $IC_{50}$  to fluoride than wild type, two-fold to FCCP and 2,4-DNP, and ten-fold and 35-fold lower to HCl and H<sub>2</sub>SO<sub>4</sub>, respectively. We tested for V-ATPase function in  $\Delta$ VMA11 yeast by monitoring the vacuolar electrochemical potential (Fig. 8B). V-ATPase is essential for maintaining the pH gradient between the cytosol and vacuole; consequently, disruption of V-ATPase disrupts this gradient (Huynh and Grinstein 2007; Maxson and Grinstein 2014). We monitored vacuolar electrochemical potential using the dye FM4-64, which enters the cells via vesicle trafficking and incorporates into the vacuolar membrane after 1 hour of exposure in normal cells. In  $\Delta$ VMA11 yeast, we found no evidence of vacuolar staining, indicating a loss of V-ATPase activity.



**Figure 8: Sensitivity of  $\Delta$ VMA11 mutation in yeast.** (A) Serial dilutions of wild type and  $\Delta$ VMA11 yeast on agar plates containing normal YPD, and YPD with NaF, HCl, FCCP,  $\text{CH}_3\text{COOH}$ , and  $\text{H}_2\text{SO}_4$ . The  $\text{IC}_{50}$ 's of each were calculated using the 24-hour liquid growth assay (data not shown). (B) Imaging of vacuolar membrane using FM4-64 dye. Cells are grown to log phase. (C) Intracellular pH of wild type and  $\Delta$ VMA11 yeast after four hours of growth, as assessed using 5(6)-CFDA. (D) Change in reactive oxygen species production in yeast grown 6 hours, in YPD or YPD with acids.

Several labs have reported that inhibition of V-ATPase results in cytoplasmic acidosis (Martinez-Munoz and Kane 2008; Diab and Kane 2013). We monitored intracellular pH at 4 hours of growth in  $\Delta$ VMA11 yeast, and found that the pH dropped from 6.2 to 5.6 (Fig. 7C). pH maintenance is an essential mechanism for acid resistance. However, disruption in intracellular pH maintenance might predictably make cells more sensitive to molecules that further disrupt intracellular pH. Conversely,  $\Delta$ VMA11 yeast were the most sensitive to strong acids HCl and  $\text{H}_2\text{SO}_4$ , which primarily alter extracellular pH. This result suggests that V-ATPase might function beyond intracellular pH maintenance in resisting acid toxicity.



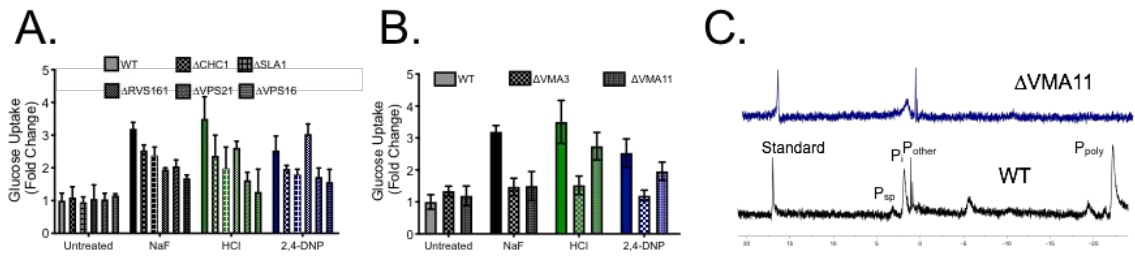
Several reports have linked vacuolar function with oxidative stress resistance, although the mechanism is still unknown (Milgrom *et al.* 2007; Diab and Kane 2013; Charoenbhakdi *et al.* 2016; Nishikiwa *et al.* 2016). Deletion of V-ATPase subunits in yeast results in hypersensitivity to a wide variety of oxidative stresses. We monitored levels of reactive oxygenated species in yeast, both under acid stress, and in V-ATPase knockout, and found that deletion of VMA11 was sufficient to increase intracellular reactive oxygenated species independent of acid stress (Fig. 7D). Addition of acids resulted in increased ROS production in wild type yeast, and even greater addition in  $\Delta$ VMA11 yeast. HCl and H<sub>2</sub>SO<sub>4</sub> did not significantly induce ROS production in wild type, but did in  $\Delta$ VMA11 yeast. While it is already well established that V-ATPase functions in pH maintenance, its role in lowering cytoplasmic ROS may also contribute to its function in general acid resistance.

***Vesicle-mediated endocytosis affects nutrient uptake during acid stress*** - Previously, our lab found that fluoride-activated acidosis disrupts the plasma membrane electrochemical gradient and initiates nutrient starvation signaling (Johnston and Strobel 2019). While yeast upregulate the expression of nutrient scavengers under fluoride stress, the transmembrane nutrient transporters are inhibited by the disrupted pH gradient. This leads to the question of how the yeast are able to uptake nutrients with inhibited protein transporters.

After vacuolar ATPase function, the next highest enriched processes involved in general acid resistance in this screen were vesicle formation, trafficking, and endocytosis. Vesicle trafficking and endocytosis are induced during acid stress (Ben-Dov and Korenstein 2013). Endocytosis is involved in both transmembrane protein recycling and nutrient uptake from the extracellular environment (Grant and Donaldson 2009; Antonescu *et al.* 2014; Hinze and Boucrot 2018). Consequently, disruption of essential genes involved in endocytosis results in cell sensitivity to nutrient depletion, particularly glucose and amino acids (Jones *et al.* 2012; Lang *et al.* 2014). Given that acid exposure decreases transmembrane nutrient transporter activity in yeast, we hypothesized that yeast utilize endocytosis during acid-induced nutrient starvation for the uptake of nutrients.

To test this hypothesis, we monitored glucose uptake during acid stress using 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-D- glucose (2-NBDG), a fluorescent glucose mimic (Fig. 9A). As expected, exposure to fluoride induced enhanced 2-NBDG uptake, as did exposure to both strong and weak acids.

Deletion of genes involved in endocytosis did not alter 2-NBDG uptake under normal conditions. However, those yeast knockout strains showed reduced uptake of 2-NBDG in the presence of fluoride or other acid, despite being more sensitized to the acid stress. The yeast strain lacking VPS16— a gene involved in the tethering and fusion of endosomes – had the most significant reduction in 2-NBDG uptake under all conditions. Together, these data support the hypothesis that endocytosis is involved in nutrient uptake during acid stress.



**Figure 9: Role of endocytosis, vesicle trafficking and V-ATPase in nutrient uptake.** Glucose uptake (monitored using the fluorescent dye 2-NBDG) after 4 hours exposure to acids in wild type (WT) and (A) endocytosis and vesicle trafficking knockouts and (B) V-ATPase subunit knockouts. (C)  $^{31}\text{P}$  NMR of wild type and  $\Delta\text{VMA11}$  yeast.

We also tested 2-NBDG uptake in V-ATPase genetic knockouts, and saw a similar reduction in intracellular 2-NBDG (Fig. 9B). This can be either due to reduced sugar uptake, or a reduction in its storage. V-ATPase is essential for regulating the pH gradient necessary for vesicle fusion and endocytosis (Geyer *et al.* 2002; Lafourcade *et al.* 2008; Maxson and Grinstein 2014). Beyond that, V-ATPase is essential for vacuolar activity, including the storage of ions. One particularly important stored ion is phosphate, which is used by the vacuole to sequester metals (Nguyen *et al.* 2019). High concentrations of stored phosphate have been demonstrated to resist oxidative stress in yeast (Lev *et al.* 2017; Johnston and Strobel 2019). We assessed phosphate storage using  $^{31}\text{P}$  NMR, and as expected, no vacuolar polyphosphate could be detected in V-ATPase mutants, and the overall concentration of intracellular phosphate was lower than wild type (Fig. 9C). In all, V-ATPase appears to function in acid resistance through oxidative stress resistance, pH maintenance, vesicle trafficking, and nutrient storage.

## Discussion

Research into the mechanics of fluoride toxicity has seen increased interest with the concern of public safety in fluoridated water. Fluoride is naturally abundant throughout our ecosystem, and additionally supplied in government-regulated water to increase our bone health. However, too much fluoride results in toxicity. While the exact cause of intracellular fluoride toxicity is unknown, the downstream stress phenotypes are well established. Fluoride elicits oxidative stress, metabolic inhibition, and intracellular acidosis (Barbier *et al.* 2010; Agalakova and Gusev 2011). This has been long presumed to be due to metalloprotein interactions with fluoride, particularly glycolytic enzymes such as enolase (Marquis 1995). However, HF is also an acid, which has underappreciated biological consequences. Here we report the screening of 5,250 non-essential *S. cerevisiae* genes for their involvement in acid resistance using a deletion library. The results demonstrate a heavy enrichment of genes involved in vacuolar function and vesicle-mediated transport for general acid resistance. Genes involved in resistance to fluoride overlapped largely with those of other acids, but had a higher enrichment in genes involved in Golgi function than the other acids.

Out of the 5,250 genes tested in this knockout screen, 32 gene deletions resulted in sensitivity to only fluoride. Of these, three genes (VPS61, YOR041C, and YDR455C), are putative open reading frames with unknown function. However, all three overlap in the genome with genes that could confer fluoride resistance. VPS61, whose deletion causes vacuolar defects, overlaps with RGP1, part of the Golgi membrane exchange factor. YOR041C overlaps with CUE5, coding a ubiquitin-binding protein involved in autophagy signaling. YDR455C overlaps with the Na<sup>+</sup>/H<sup>+</sup> exchanger NHX, which itself conferred resistance to fluoride toxicity and is required for osmotolerance. Other genes involved uniquely in fluoride tolerance were the copper transporter CCC2, the cytochrome c oxidase gene COX20, stress transcription factor CRZ1, and the DNA repair gene IES6. Each of these were not part of a conserved pathway of genes related to fluoride resistance, but most probably function in general stress resistance. Fluoride is well established to cause metal imbalance, DNA damage, oxidative stress, and metabolic arrest; expression of these genes would counteract these effects (He and Chen 2006; Fina *et al.* 2014; Johnston and Strobel 2019).

The most significantly enriched pathway unique to fluoride resistance was Golgi function and vesicle-mediated transport. Among these were genes involved in endocytosis and cell surface maintenance, including

ERG24, HOF1, RVS167, VPS9, and VRP1. Also unique to fluoride were subunits of the endocytosis tethering complexes ESCRT and CORVET. Endocytosis was found to confer resistance against all acids tested in this report. Why these five genes caused sensitivity only to fluoride, is not immediately clear. Fluoride has previously been reported to selectively inhibit vesicle trafficking, both through metal- and G-protein-interactions (Matsuo *et al.* 1998; Stow and Heimann 1998; Taraschi *et al.* 2001; Barbier *et al.* 2010). As such, it could be that these sets of genes are particularly sensitized to fluoride as opposed to other acids. Supporting the hypothesis that vesicle-transport is most sensitized to fluoride exposure, many genes essential for SNARE and Golgi function conferred resistance to only fluoride. Metallo-fluoride also reversibly disrupts Golgi stacking and inhibits essential Golgi GTPases (Finazzi *et al.* 1994; Back *et al.* 2004). One particular known target, Arf1p GTPase, was among the genetic deletions that caused sensitivity only to fluoride toxicity (Lanoix *et al.* 2001). Given that these genes are unique in affecting fluoride resistance and there is scientific precedent that metallo-fluoride alters their activity, Golgi and vesicular trafficking are likely specific targets of fluoride.

Genes involved in general protein turnover, including peroxisome function, amino acid synthesis, and ribosomal function, are involved in fluoride resistance. Stress, in general, is rescued by functional protein turnover, which can degrade inhibited or counterproductive proteins and synthesize proteins that combat stress. However, fluoride is known to bind to and inhibit ribosomes (Ravel *et al.* 1966; Vesco *et al.* 1970; Hardesty *et al.* 1973). Fluoride also elicits oxidative stress, which independently halts translation (Shenton *et al.* 2006). Given that acid stress is rescued by protein turnover, non-essential genes that aid in protein synthesis could provide significant tolerance to fluoride.

The majority of genes that conferred significant resistance to fluoride toxicity also conferred resistance to acids lacking high metal affinity. These genes were largely enriched for involvement in V-ATPase and vesicle-mediated transport. Both of these have a multitude of functions that could potentially aid in acid resistance. For instance, V-ATPase functions in pH maintenance, endocytosis regulation, and nutrient storage (Maxson and Grinstein 2014). We also demonstrate that inhibition of V-ATPase resulted in an increase of intracellular ROS independent of additional stressors. As many acids – including fluoride – cause oxidative stress, loss of ROS maintenance would predictably enhance toxicity. Endocytosis is also involved in many processes, including cell surface turnover and protein recycling (Goode *et al.* 2015). Endocytosis has been

previously reported to confer resistance to acid stress, and conversely, both alkaline and acid stress partially inhibit endocytosis (Sandvig *et al.* 1987; Pereira *et al.* 2012; Ben-Dov and Korenstein 2013). We report here that endocytosis is involved in the uptake of nutrients during pH disruption. Given that many acids facilitate pH disruption along the plasma membrane, one might predict that endocytosis is involved in general acid resistance.

Due to the limitations of a genetic knockout library, we are not able to assess the involvement of essential genes in general acid resistance, such as the presumed key target of fluoride enolase. However, we are able to investigate the role of nonessential genes involved in metabolism to identify the overlap between fluoride and general acid toxicity resistance. These nonessential genes involved in metabolism did not significantly impact fluoride, or general acid resistance.

Fluoride toxicity has long been attributed to metal interactions *in vivo*. While metal binding is undoubtedly a factor, the role of acid stress has been underappreciated. Here, we report that the majority of nonessential genes involved in fluoride resistance are also involved in general acid resistance, particularly pH maintenance and vesicle transport. Acid stress is commonly encountered in the wild. As such, it would confer a significant evolutionary advantage for organisms to retain a widespread resistance mechanism. Overall, these data suggest that a significant factor in fluoride toxicity can be attributed to its properties as an acid.

## Chapter 5: A novel role of pore 1 in eukaryotic fluoride transporters for general acid resistance

*This section represents a manuscript in preparation for publication. Plasmids expressing plant FEX were prepared by Susan L. Tausta. Plasmids expressing animal FEX were prepared by Taylor Sells. Early liposome assays and FEX purification were conducted by myself and Martin G. Peverelli. All other experiments were conducted by myself. The manuscript and figures were prepared by myself.*

### **Abstract**

Fluoride transporters are widely conserved across prokaryotes, archaea, and eukaryotes. In prokaryotes and archaea, fluoride transporters are homodimer transmembrane channels that rapidly excrete intracellular fluoride through two active pores. Conversely, eukaryotic fluoride transporters form a single polypeptide pseudodimer and remove intracellular fluoride at a rate more than 2,000-fold slower than prokaryotic exporters. This function is attributed to pore 2, whereas pore 1 is conserved in eukaryotes but has no known activity. Here, we report that pore 1 is an essential component of eukaryotic fluoride transporters in conferring resistance to extracellular acidification, independent of fluoride. The eukaryotic fluoride transporter was found to alter the pH both *in vivo* and in reconstituted liposomes in the presence of a proton gradient. Collectively, our data suggests that eukaryotic fluoride transporters act not as channels, but rather exchange transporters for fluoride and either a proton or hydroxide. Our findings highlight a mechanism of stress resistance conserved in eukaryotes by which a transporter confers resistance to two types of ion stress, both fluoride and pH.

### **Introduction**

All organisms use proteins to regulate the flow of molecules into and out of cells. Most commonly, organisms express specialized proteins that localize to the plasma membrane and directly facilitate the shuttling of molecules across the lipid bilayer. These proteins can be grouped into the general classes of channels and transporters. Channels are water-filled pores that allow for the passive movement of molecules along their chemical or electrical gradient (Albert *et al.* 2002). Passage through a channel is fast, often with millions of substrates travelling across the membrane per second. Unlike simple membrane pores, most channels are gated so that substrates only travel through under the appropriate stimuli. Channels do not bind their substrate, nor change their structure during activity. Conversely, transporters bind substrates at multiple

sites, and often change their structure during activity (Albert *et al.* 2002). As a consequence, the rate of passage through a transporter is much slower than through a channel. Transporters do not always act passively. Pumps utilize either light, chemical energy, or the electrochemical gradient to shuttle a substrate against its gradient. Similarly, coupled transporters transport multiple substrates – either together or in opposing directions – wherein the movement of one substrate along its gradient drives the movement of the other substrate against its gradient. Transporters typically feature at least two gates, which regulate substrate shuttling on both sides of the membrane.

As our collective scientific knowledge advances, the distinction between channels and transporters blurs. Transporters are tightly regulated by gating. Fundamentally, disruption of gating in transporters would result in channel-like behavior (Ashcroft *et al.* 2009). This has been observed in the protein AE1, in which four single-point mutations each shift AE1 activity from a cation transporter to a cation channel (Ellory *et al.* 2009). Given that small changes to a protein can illicit significant shifts in its activity, many families of ion channels or transporters feature members of both groups. One such example is the chloride channel (Clc) family, found in both animals and bacteria (Jensch *et al.* 1999). The Clc family contains several chloride channels, as well as chloride/proton antiporters. Recently, a member of the Clc family, CLC<sup>F</sup>, was identified in bacteria to act as a fluoride/proton antiporter (Brammer *et al.* 2014). This protein is one of two known to remove fluoride from bacteria, the other being a fluoride channel (Fluc).

Fluc is a member of the Fluc family of fluoride channels. Fluc is a voltage-gated channel that rapidly exports fluoride at a rate of 10 million fluoride ions per second (Stockbridge *et al.* 2013). Fluc is composed of four transmembrane helices, and *in vivo* binds to another Fluc to form a homodimer with two pores. Eukaryotes, including microbes, plants, and sea sponges, express the fluoride exporter (FEX) from the Fluc family. Fluoride efflux by FEX is much slower than Fluc, at 4,000 ions per second (Peverelli and Strobel, unpublished). FEX is composed of nine transmembrane helices, or two domains comprised of four transmembrane helices and one linker helix. FEX forms a pseudodimer with two pores. Unlike Fluc, only pore 2 of FEX was found to participate in fluoride efflux (Berbasova *et al.* 2017). Despite its lack of known function, pore 1 is highly conserved across eukaryotes. This suggests an import, undiscovered role for FEX pore 1 activity.

Here, we report a novel role for pore 1 of eukaryotic FEX in conferring resistance to extracellular acidification. The expression of FEX in yeast allows growth in acidic environments a full pH unit below the growth capabilities of yeast lacking FEX. Prior to this investigation, the only known function of Fluc and FEX was to remove intracellular fluoride. This new role of FEX has implications both on our knowledge of protein transporter capabilities, and acid tolerance mechanics. Given that FEX is conserved across eukaryotes including plants, many animals, and possibly humans, this knowledge of FEX activity has far reaching applications into how organisms cope with our increasingly acidic environment.

### **Materials and Methods**

Strains, media, and reagents: The yeast strain used in this paper is BY4741, and the subsequent FEX DKO strain was generated by Li *et al.* 2013 using the hphMX4 and kanMX6 resistance cassettes (*MATa his3Δ1 leu2Δ0 ura3Δ0 FEX1Δ::kanMX6 FEX2Δ::hphMX*). Plasmids were constructed on pRSFTPIL vectors and transformed using standard procedure (pRSFTPIL vectors contain ampicillin resistance and URA markers). Unless otherwise noted, all reagents were commercially available. Yeast were grown in YPD buffer, made up of 1 g yeast peptone (Becton, Dickinson and Co.), 0.5 g yeast extract (Becton, Dickinson and Co.), 50 μL of 1% adenine (Sigma), and 2.5 mL of 40% glucose (Sigma) per 50 mL total volume in water.

Liquid growth assay: Liquid growth assays were conducted over 24 hours at starting O.D. 0.1 in 1 mL media, as outlined in Li *et al.* 2013. Sodium fluoride (Sigma Aldrich), HCl (Sigma Aldrich), H<sub>2</sub>SO<sub>4</sub> (Sigma Aldrich), 2,4-DNP (Sigma Aldrich), and acetic acid (J.T. Baker) were used in the reported experiments.

Intracellular and extracellular pH: For monitoring pH, cells were grown for the denoted time in 2 mL YPD +/- acid, then harvested using centrifugation. The media was separated from the cells, and a pH electrode was used to assess the extracellular pH. Intracellular pH was determined using 5(6)-CFDA dye under its standard protocol.

Bioinformatics: Alignment and analysis of fluoride transporters was conducted using Clustal Omega and WebLogo. The heat maps were constructed by using the method outlined by Requiao *et al.* 2017.

Mutating FEX: FEX was mutated on a pRSFTPIL vector using overlapping primers containing the mutated region. The mutated plasmids were constructed via PCR, then purified and sequenced to confirm the



mutation. Plasmids were then transformed into yeast using the standard protocol. A list of primers used can be found in the Supplementary Information.

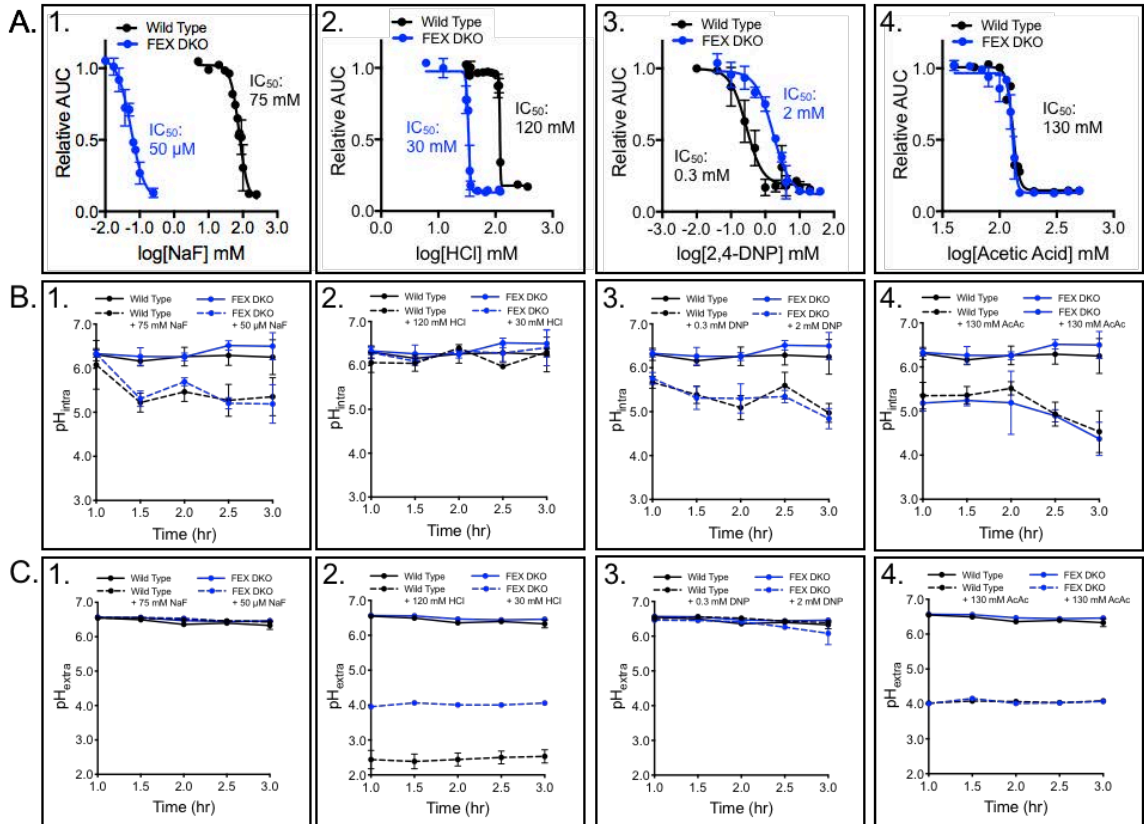
Protein purification: FEX was purified from yeast transformed with a pEGH vector containing GST-labelled *S. cerevisiae* FEX1. Expression was induced using 2% raffinose, 2% galactose, and 0.1% dextrose. After overnight growth, yeast were suspended in media containing 1 tablet cOmplete protease inhibitor, 1 mM EDTA, and 50 mL ice cold PBS, before passing through a microfluidizer in lysis buffer (1 mM DTT, 1 mM PMSF, 1 mM EDTA, and DNase I into 150 mL ice cold PBS). The lysate was rocked in the cold room for two hours with an additional 1.2% decyl maltoside (DM). After spinning 30,000 g for 15 minutes at 4°C, the supernatant was passed through an FPLC equipped with a GST-trap HP column (Buffer A – 0.2% DM in ice cold PBS; Buffer B – 0.2% DM, 20 mM Tris, 150 mM NaCl, 40 mM reduced glutathione in water, pH 8.0). After elution from the column, FEX was concentrated using a 30 kDa centrifugal concentrator, then ran through FPLC again using a HiTrap desalting column. FEX was further purified by passing through a chromatography column loaded with GSH-resin and Buffer A, then again spun in a 30 kDa concentrator. The concentration and purity of FEX was monitored in between steps using western blotting, Coomassie gels, and a BCA assay kit. After purification, FEX was flash-frozen and stored at -80°C until use.

Liposome reconstitution: Liposomes were prepared as 10 mg/mL lipid from *E. coli* lipid extract (Avanti) (for protein free), or 10 mg/mL lipid + 5 µg FEX (for proteoliposomes). Liposomes were dissolved in the internal media (15 mM HEPES, 32.4 µM Na<sub>2</sub>SO<sub>4</sub>, and buffered to the denoted pH using NaOH), plus an additional 35 mM CHAPS. After dissolving, the CHAPS was removed with two days of dialysis in internal buffer at 4°C. Liposomes were then frozen/thawed at least four times, and sonicated briefly. Liposomes were frozen at -80°C until use, and kept cold at all times. Immediately before functional assays, liposomes were extruded 19 times through a 400-nm Whatman nuclepore track-etched membrane filter, then centrifuged through a Sephadex G-50 column containing a 2 mL slurry of G50-sephadex resin and external buffer (1 mM HEPES, buffered to the denoted pH using H<sub>2</sub>SO<sub>4</sub>) at 4°C. 120 µL of proteoliposomes were then added to 1.78 mL external buffer, and ion flux was monitored using either a fluoride or proton electrode.

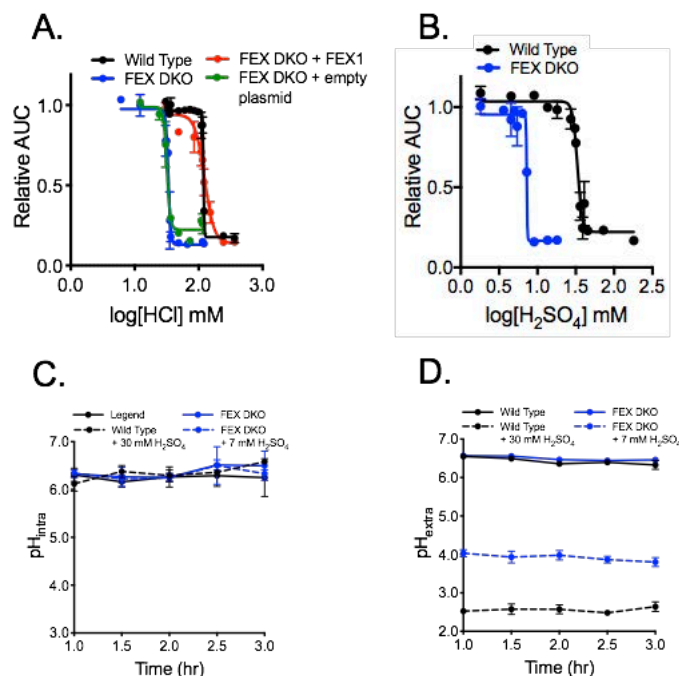
## Results

***Yeast fluoride exporter confers acid resistance that is dependent on pH gradient*** - Yeast express two copies of the fluoride exporter, FEX1 and FEX2. These exporters are functionally redundant, and expression

of one is enough to rescue from fluoride toxicity in a single deletion strain (Li *et al.* 2013). However, deletion of both FEX1 and FEX2 (double knockout, or DKO), results in over 1,000-fold sensitivity to fluoride (Fig. 1A-1). Exposure to fluoride at over 50  $\mu$ M in FEX DKO, and 75 mM in wildtype, does not alter extracellular pH, but does cause intracellular acidification (Fig. 1B-1, 1C-1). Here we report the novel finding that deletion of both copies of FEX also results in 4-fold increased sensitivity to extracellular acidification by HCl or H<sub>2</sub>SO<sub>4</sub> (Fig. 1A-2, Suppl. 1B). This sensitivity is dependent on the pH gradient; yeast are resistant to intracellular acidification by 6-fold with the deletion of FEX1 and FEX2 (Fig. 1A-3). This effect is not dependent on the presence of fluoride, as the acids used contained no traceable fluoride contamination (data not shown). Furthermore, abolition of the pH gradient by causing acidity on both the inside and outside results in no advantage from FEX expression (Fig. 1A-4). Transformation of FEX DKO yeast with a plasmid containing FEX restored acid resistance to that of wild type, indicating that the effect is due to FEX and not the strain (Fig. 2A).



**Figure 1: FEX confers acid resistance depending on pH gradient.** Represented here are (A) liquid growth assays over 24 hours, (B) intracellular pH over 3 hours, and (C) extracellular pH over 3 hours in wild type and FEX DKO yeast. Yeast are either grown in plain YPD media, or in (1) NaF, (2) HCl, (3) 2,4-DNP, or (4) acetic acid at their respective  $IC_{50}$  concentrations.

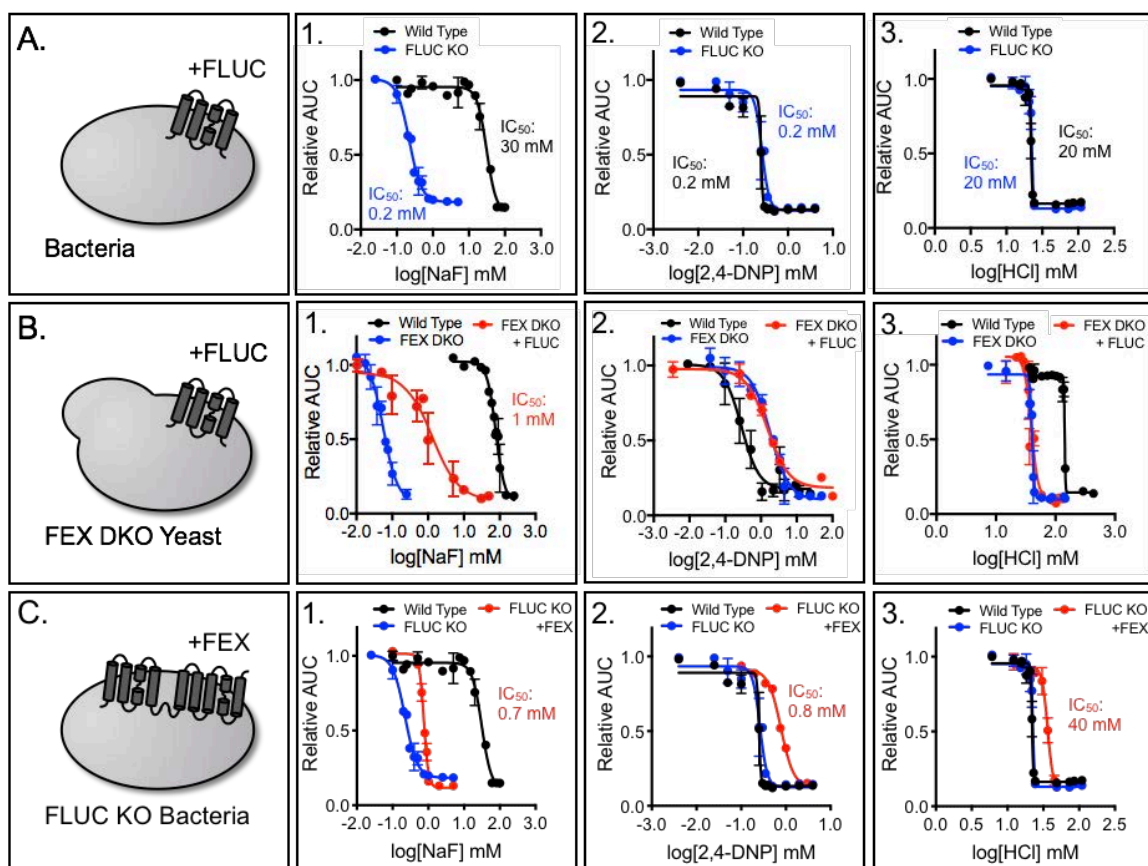


**Figure 2: Acid resistance by FEX is not dependent on Cl.** Represented here are (A) liquid growth assays over 24 hours of Wild Type, FEX DKO, FEX DKO + empty plasmid, or FEX DKO + FEX1 in increasing exposure to HCl. (B) liquid growth assays over 24 hours of Wild Type and FEX DKO in increasing exposure to H<sub>2</sub>SO<sub>4</sub>. (C) Intracellular and (D) Extracellular pH of cells exposed to H<sub>2</sub>SO<sub>4</sub> for 3 hours at their respective IC<sub>50</sub> concentrations.

***Plant and animal, but not bacterial, fluoride exporters confer acid resistance*** - We next looked for acid tolerance in other members of the Fluc family. Deletion of Fluc in *E. coli* resulted in a 150-fold loss in fluoride resistance; however, it had no significant impact on bacterial sensitivity to intracellular and extracellular acidification (Fig. 3A). Likewise, insertion of Fluc into FEX DKO *S. cerevisiae* conferred a 20-fold increase in fluoride resistance, but did not change yeast sensitivity to acid (Fig. 3B). However, insertion of FEX1 into Fluc knockout *E. coli* resulted in both 4-fold increase in fluoride resistance, as well as a 2- or 4-fold increase in acid resistance (Fig. 3C). Unlike yeast, bacteria had increased resistance to both intracellular and extracellular acidity while expressing FEX1. There are several key differences between prokaryotic and eukaryotic transmembrane protein insertion, including post-translational modifications, glycosylation,

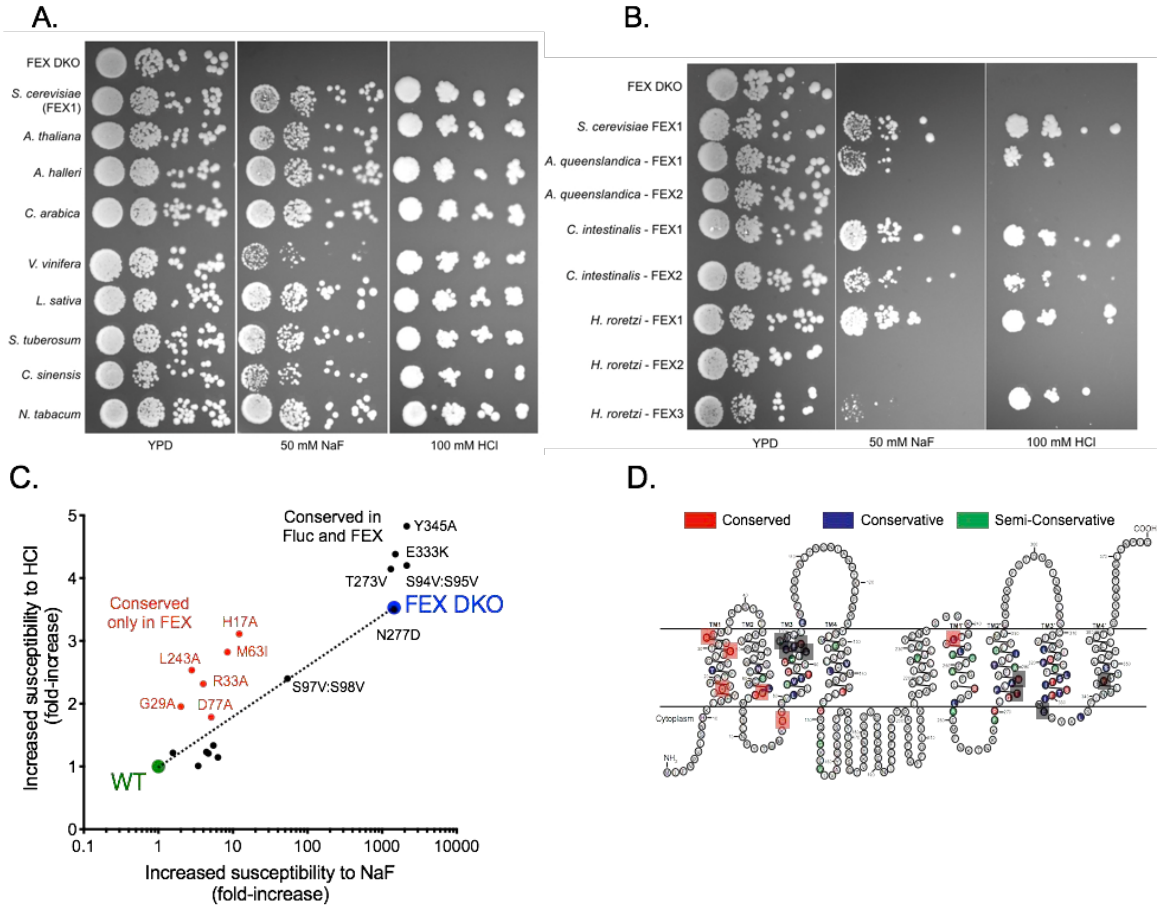
expression levels, surface charge, and lipid composition; all of which could impact FEX1 activity. Collectively, this data demonstrates that FEX but not Fluc confers acid resistance across yeast and bacteria.

The Fluc family is conserved across many branches of life, including plants and animals. Using bioinformatics, we identified putative fluoride transporters in 8 species of plants, and 4 species of sea sponges and tunicates (data not shown). These fluoride transporters were then cloned onto a high expression plasmid and transformed into FEX DKO yeast. We screened for both fluoride and hydrochloric acid tolerance using a serial dilution assay (Fig. 4A-B). All putative plant fluoride transporters tested conferred significant resistance to both fluoride and hydrochloric acid, while six of the eight putative animal transporters conferred both fluoride and hydrochloric acid resistance. Putative eukaryotic transporters that conferred fluoride resistance always conferred acid resistance as well.



**Figure 3: Fluc and FEX activity in acid resistance.** Liquid growth assays over 24 hours of (A) wild type or Fluc knockout bacteria, (B) wild type or FEX DKO yeast +/- Fluc, or (C) wild type of Fluc knockout bacteria +/- FEX at starting O.D. 0.1 in 1 mL YPD (yeast) or LB (bacteria) broth containing increasing (1) fluoride, (2) 2,4-DNP, or (3) HCl. Data is represented as the change in the total area under the curve (AUC) of growth, as outlined in materials in methods.

***Both pore 1 and pore 2 are necessary for resistance to extracellular acids*** - Given that eukaryotic, but not prokaryotic members of the Fluc family respond to extracellular acidification, comparison of their gene sequences provides us with valuable insight into the amino acids responsible for this activity. Alignment of eukaryotic FEX and its comparison to Fluc has been reported by our lab previously (Berbasova *et al.* 2017). In short, sequence similarity between eukaryotic FEX is low (25-30% identity). Eukaryotic FEX is most similar to bacterial Fluc in the regions corresponding to pore 2 (41% identity), and less similar in regions corresponding to pore 1 (29% identity).

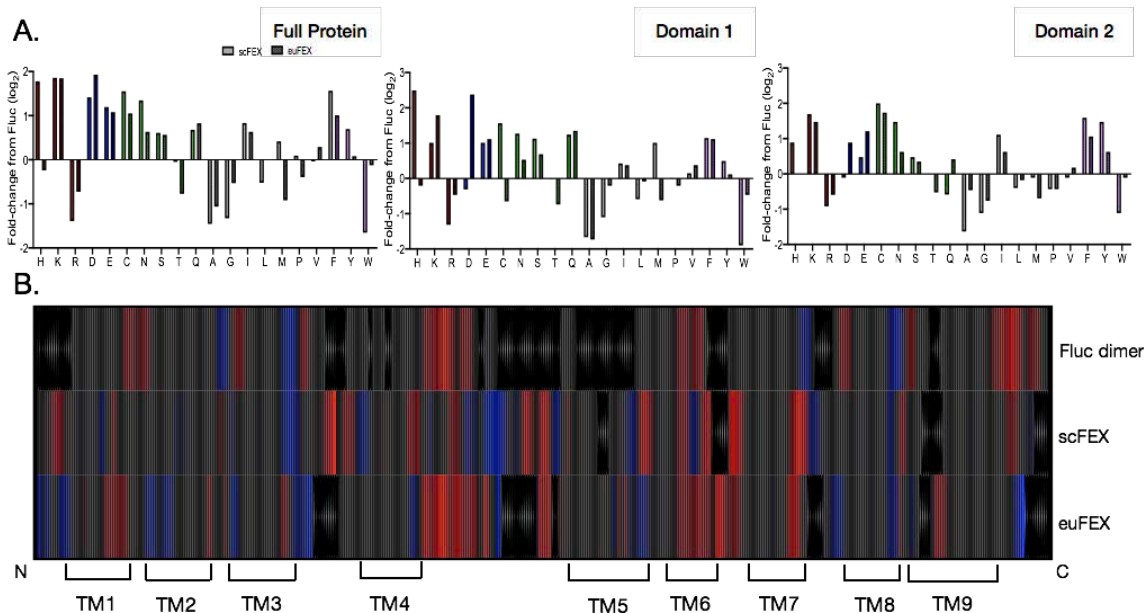


**Figure 4: Acid resistance across eukaryotic FEX.** Serial dilutions of FEX DKO yeast in plain YPD-agar, or with 50 mM NaF or 100 mM HCl. Yeast are transformed with either a blank plasmid (FEX DKO), a plasmid containing FEX1 from yeast (*S. cerevisiae* FEX1), or a plasmid containing a putative FEX from (A) plants or (B) animals, as predicted using gene consensus alignment. (C) Fold-increase in susceptibility either fluoride or hydrochloric acid by making the denoted changes to FEX in yeast. Black dots are residues conserved in both Fluc and FEX, while red dots are residues conserved in only FEX. (D) Diagram of yeast FEX1, demonstrating where each amino acid is predicted to localize along the plasma membrane (Smith *et al.* 2015). In red, blue, and green are conserved, conservative, and semi-conservative residues across eukaryotic FEX. Red and grey boxes denote regions that confer significant ion sensitivity and are conserved in FEX, or Fluc and FEX, respectively.

Alignment of the 14 eukaryotic fluoride transporters demonstrate localized conservation along the transmembrane helices rather than the cytosolic or extracellular regions. As the regions outside of lipids are where other proteins could interact with FEX, the lack of amino acid conservation in these regions argues against a model in which an interacting partner confers acid resistance.

We next generated a “typical” eukaryotic FEX sequence based off most frequently occurring amino acids. Compared to Fluc, this “euFEX” had an overall 2- to 4-fold increase in abundance of negatively charged amino acids, a 3-fold increase in the positively charged amino acids H and K, a 2-fold decrease in E, and a 2- to 3-fold increase in the polar amino acids C, N, and S (Fig. 5A). Conversely, there was a 2-fold decrease in the occurrence of uncharged residues G and A. These trends were largely consistent comparing the change of amino acid abundance between the Pore 1 region of euFEX versus Fluc. The changes in amino acid composition led to an overall shift in charged regions of FEX, where areas near the edges of the helical regions were more positively charged than in Fluc (Fig. 5B). As these regions are where gating would occur in transporters, this hints at the presence of a charge barrier at the surfaces of FEX that are not present in Fluc.



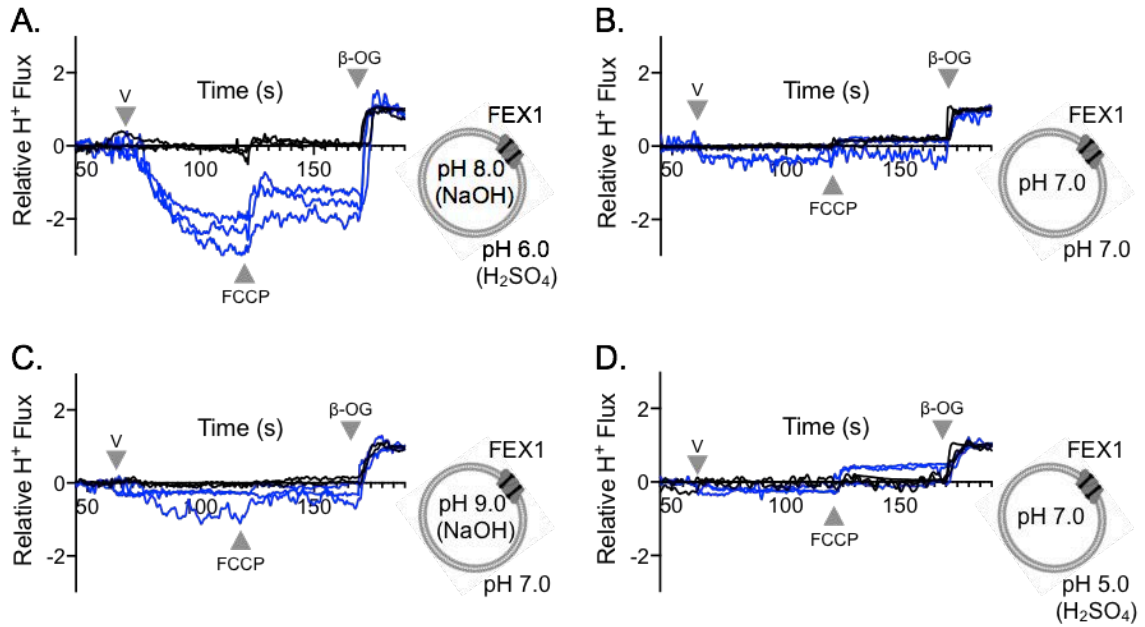


**Figure 5: Comparison of FEX sequence to Fluc.** (A) Fold change in percent abundance of amino acids in yeast FEX (scFEX) or the conserved sequenced of eukaryotic FEX (euFEX) compared with Fluc sequences. Color coding is red: positively charged, blue: negatively charged, green: polar, and purple: aromatic. (B) Heat map of the total charged regions of Fluc, yeast FEX, or the conserved eukaryotic FEX. Blue denotes more negatively charged regions, while more positively charged regions are in red. Regions that are black with a white stripe denote gaps in the amino acid alignment.

In order to isolate individual amino acids critical for FEX function, we performed a series of mutagenesis assays. First, we mutated single amino acids conserved in pore 2 of both Fluc and eukaryotic FEX (Fig. 4C). Mutation of these amino acids equally reduced both fluoride, and acid tolerance on a log scale. This argues that pore 2 is critical for both functions. We then performed single amino acid mutations to residues that were conserved in only eukaryotic FEX, altering them to their amino acid counterpart expressed in Fluc. 29 residues were highly conserved in eukaryotic FEX but not prokaryotic Fluc. We found that of particular importance were H17A, M63I, L243A, and R33A. These amino acids are predicted to be in within the helical regions of pore 1. When plotted on a log scale, mutations to pore 1 affected the fold-change in acid tolerance more than the fold-change in fluoride tolerance. Together, the results of mutagenesis assay suggest that pore 2 is essential for both fluoride and acid tolerance, while pore 1 is essential for acid tolerance. The feature of

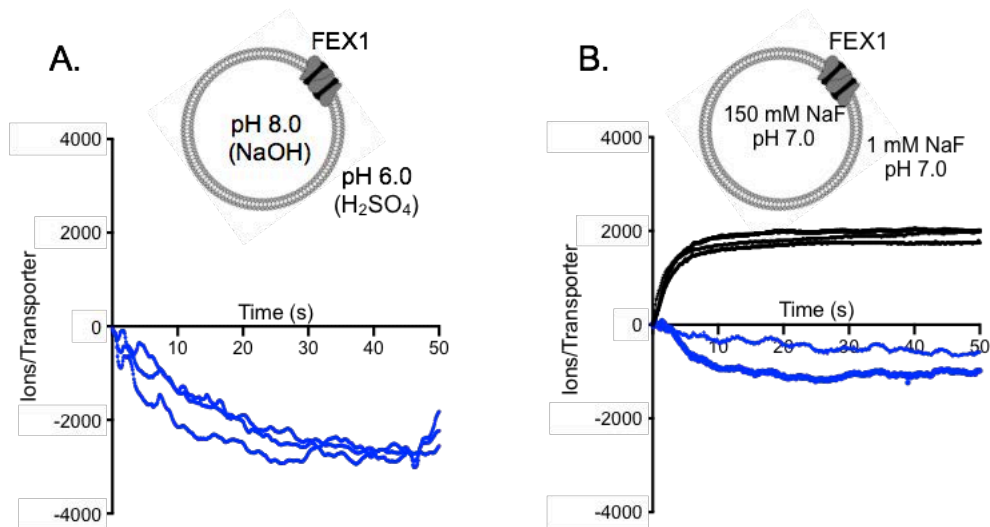
multiple amino acids participating in substrate recognition supports the notion that eukaryotic FEX is acting as a transporter.

**Activity of FEX in reconstituted liposomes is consistent with either H<sup>+</sup> or OH<sup>-</sup> transport** - To assess the ability of eukaryotic FEX to rescue from extracellular acid stress, we reconstituted yeast FEX1 into liposomes and performed an ion flux assay using a H<sup>+</sup> electrode. FEX1 in liposomes with an internal pH of 8.0 and external pH of 6.0 resulted in a decrease of voltage in the external buffer, indicating either H<sup>+</sup> influx or OH<sup>-</sup> efflux (Fig. 6A). This was calculated to occur at an approximate rate of  $3.7 \times 10^3$  ions/sec, for 2,600 total ions during flux. When we abolished the pH gradient, ion flux was also eliminated (Fig. 6B). This supports our *in vivo* data that FEX1 is able to counteract an acidic environment independent of fluoride.



**Figure 6: Proton flux in eukaryotic FEX.** Liposomal functional assays of FEX1 in 15 mM HEPES (inside), 1 mM HEPES (outside), at (A) pH<sub>in</sub> 8.0, pH<sub>out</sub> 6.0, (B) pH<sub>in</sub> 7.0, pH<sub>out</sub> 7.0, (C) pH<sub>in</sub> 9.0, pH<sub>out</sub> 7.0, and (D) pH<sub>in</sub> 7.0, pH<sub>out</sub> 5.0. Proton flux was assessed by a H<sup>+</sup> electrode that measured the change in voltage over time during the assay. Blue lines denote liposomes+FEX1, while black lines are liposomes alone. The grey arrows denote the timepoints in which valinomycin, FCCP, and β-OG were added, respectively.

FEX1 can alter the extracellular pH either through efflux of  $\text{OH}^-$  or influx of  $\text{H}^+$ . To our knowledge, no  $\text{OH}^-$  electrode exists, and we therefore cannot directly measure  $\text{OH}^-$  flux. Instead, we compared FEX1 activity with a more alkaline liposome, versus a more acidic environment (Fig. 6C-D). FEX1 reconstituted in liposomes with an alkaline internal environment had a 2-fold decreased rate of efflux, while those in an acidic external environment had a 9-fold decreased rate of efflux compared with FEX1 in liposomes with both an alkaline internal and acidic external environment. Given that FEX1 had more activity under an alkaline environment,  $\text{OH}^-$  most likely passes through FEX1 more efficiently than  $\text{H}^+$ . Nonetheless, as gradients on both sides of the liposome were necessary, most likely both  $\text{H}^+$  and  $\text{OH}^-$  are being transported. Furthermore, voltage change in liposomes at neutral pH with an internal gradient of 150 mM NaF was consistent with 2:1  $\text{F}^-/\text{H}^+$  exchange, and the total ion flux would suggest a 2:1  $\text{OH}^-/\text{H}^+$  exchange in the presence of a pH gradient (Fig. 7). This is consistent with FEX exporting a  $\text{F}^-$  ion rather than  $\text{OH}^-$  in the presence of fluoride. Given that two distinct pores are involved, we hypothesize pore 1 imports  $\text{H}^+$ , while pore 2 exports  $\text{OH}^-$  under acidic environments.



**Figure 7: Total proton flux in eukaryotic FEX.** Liposomal functional assays of FEX1 in 15 mM HEPES (inside), 1 mM HEPES (outside), at (A)  $\text{pH}_{\text{in}}$  8.0,  $\text{pH}_{\text{out}}$  6.0, (B)  $\text{pH}_{\text{in}}$  7.0 and 150 mM NaF (inside),  $\text{pH}_{\text{out}}$  7.0 and 1 mM NaF (outside). Assay is plotted immediately following valinomycin treatment, and is adjusted for total ion flux per transporters reconstituted in liposomes. Blue lines denote flux measured with an  $\text{H}^+$  electrode, while black lines denote flux measured with a  $\text{F}^-$  electrode.

## Discussion

Here we report the first finding of the eukaryotic FEX protein rescuing from extracellular acidification. Prior to this report, FEX was only known to remove intracellular fluoride from cells. However, deletion of FEX from yeast resulted in sensitivity to fluoride not only at a lower extracellular concentration, but also a lower intracellular concentration, suggesting that FEX has a role in toxicity tolerance beyond fluoride (Johnston and Strobel 2019). FEX is a member of the same protein family as the bacterial channel Fluc, yet has a rate of fluoride efflux more in line with typical transmembrane transporters (Peverelli and Strobel, unpublished). FEX folds to form two pores, of which only one pore has been previously attributed with ion flux (Berbasova *et al.* 2017). Here we find that the other pore is not vestigial, but rather functions in the transport of either H<sup>+</sup> or OH<sup>-</sup>. The two pores work together during acid stress, and together confer four-fold rescue from extracellular acidification.

The other member of the fluoride transport family, Fluc, is well characterized. The directionality of Fluc activity is regulated by the negative membrane potential in prokaryotes, which allows for the efflux of fluoride even in highly concentrated environments (Ji *et al.* 2014). This efflux is consistent with the rate of a typical channel, at several millions of ions per second (Stockbridge *et al.* 2013). However, other reports have found that the altering of gates or communication between gates along proteins can shift their activity from channels to proteins or vice versa (Ellory *et al.* 2008; Finkelstein 2009; Gadsby *et al.* 2009). This creates a precedent that subtle residue alterations between members of a protein family could result in a shift from channel-like to transporter-like activity.

The Fluc family of fluoride channels has over 8,000 homologs across archaea, bacteria, and eukaryotes (Li *et al.* 2013). Many archaea and bacteria also express the Clc family protein CLC<sup>F</sup>, which functions as a F<sup>-</sup>/H<sup>+</sup> exchanger (Stockbridge *et al.* 2012). Eukaryotes, on the other hand, express FEX. FEX has been identified in fungi, plants, and even several species of animals, including sea sponges and tunicates (Li *et al.* 2013). Sea sponges are of great concern to climate change researchers, as it has been reported that sponges optimally erode corals under acidic conditions (Wisshak *et al.* 2012; Wisshak *et al.* 2014; Achlatis *et al.* 2017). Given our research, it is possible that the presence of FEX in sea sponges aids in their ability to proliferate and metabolize corals in acidic water. Humans are predicted to express FEX based off the variation

of fluoride tolerance across tissues, but no protein has so far been identified. This identification of a novel role for FEX provides a new avenue for identifying human fluoride exporters.

We are limited from further characterizing acid tolerance by FEX because of liposomal leakage and lack of technology. We found that leakage of protons across liposomes increased proportionally with the fluoride or pH gradient (data not shown). The data reported here represents our most optimized procedure, in which the outside and inside concentration of  $\text{OH}^-$  and  $\text{H}^+$  was balanced by averaging pH 7.0 across the gradient. The more we deviated from this average, the greater the leakage. This was also the case with fluoride; addition of a fluoride gradient greatly enhanced proton leakage. Consequently, we were not able to accurately compare the rate of pH change with a 150 mM fluoride gradient, as is done typically (Stockbridge *et al.* 2012; Stockbridge *et al.* 2013; Peverelli and Strobel, unpublished). Furthermore, we were unable to completely eliminate  $\text{H}^+$  or  $\text{OH}^-$  from either side of the liposome due to leakage constraints. To our knowledge there is no electrode that distinguishes the pH change from  $\text{H}^+$  influx, versus  $\text{OH}^-$  efflux. We are therefore unable to classify pore 1 as a  $\text{H}^+$  importer or  $\text{OH}^-$  exporter. However, we know that the residues of pore 2 allow for the efflux of  $\text{F}^-$ .  $\text{OH}^-$  and  $\text{F}^-$  are very similar in size, and therefore we predict that pore 2 functions in  $\text{OH}^-$  efflux during acid stress.

Fluoride is present throughout the environment, and is the most toxic to organisms when in an acidic context. It therefore would confer a significant evolutionary advantage for a protein that rescues from fluoride to co-rescue from an acidic environment. Given that FEX is not necessarily expressed across all eukaryotes, this finding furthers both our knowledge of the mechanics of pH tolerance, as well as provides us with a future avenue for the exploration of differing pH tolerance across organisms.

## Chapter 6: Conclusion and Future Directions

Exposure to high concentrations of fluoride is well established to cause a broad array of toxicity phenotypes, including oxidative stress, metabolic arrest, DNA damage, and eventual apoptosis. The mechanism behind these phenotypes has never been established, but has long presumed to due to the high affinity between fluoride and metals.

Here, I reported that the acidic properties of HF represent a large portion of the toxicity response in yeast. This response is particularly enriched for genes linked to nutrient starvation apoptosis and glucose, phosphate, and amino acid uptake. I also found that yeast can adapt to detoxify fluoride by sharing nutrients, reducing metabolism, and acidifying their cytosolic components. The acidification in particular is beneficial to fluoride resistance, as fluoride becomes protonated and excreted from the cell without the need of a protein channel. In nature, yeast express the protein channel FEX. I found that FEX both excretes fluoride and confers resistance to acid. Based on the amino acid mutagenesis and liposome assays, I predict this is due to FEX's ability to exchange both  $F^-/H^+$  and  $OH^-/H^+$ . I also found this function is conserved across eukaryotes, and confers significant resistance to extracellular acid stress.

Many questions arise from these studies. The principle question remains: what is the exact mechanism of fluoride toxicity? We are now closer to this answer by understanding that most classical phenotypes of fluoride toxicity are due to intracellular acidification. However, the exact mechanism by which fluoride elicits stress signaling and enhances intracellular acidification remains a mystery. A key limitation to my studies is not being able to remove essential processes, such as protein synthesis, cell surface construction, and metabolism. A systematic investigation of the order in which each of these processes are inhibited would prove extremely useful in our understanding of fluoride toxicity. Eventually, we need to identify the event immediately upstream of intracellular acidification, as this process is most likely the key mechanism of fluoride toxicity.

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