Characterization of a patient-derived variant of GPX4 for precision therapy

Hengrui Liu1, Farhad Forouhar2, Tobias Seibt3, Russell Saneto4,5, Kristen Wigby6,7, Jennifer Friedman6,7,8,9, Xin Xia10, Mikhail S. Shchepinov11, Sanath Kumar Ramesh12, Marcus Conrad13,14 and Brent R. Stockwell1,2,10,15

Glutathione peroxidase 4 (GPX4), as the only enzyme in mammals capable of reducing esterified phospholipid hydroperoxides within a cellular context, protects cells from ferroptosis. We identified a homozygous point mutation in the GPX4 gene, resulting in an R152H coding mutation, in three patients with Sedaghatian-type spondylometaphyseal dysplasia. Using structure-based analyses and cell models, including patient fibroblasts, of this variant, we found that the missense variant destabilized a critical loop, which disrupted the active site and caused a substantial loss of enzymatic function. We also found that the R152H variant of GPX4 is less susceptible to degradation, revealing the degradation mechanism of the GPX4 protein. Proof-of-concept therapeutic treatments, which overcome the impaired R152H GPX4 activity, including selenium supplementation, selective antioxidants and a deuterated polyunsaturated fatty acid were identified. In addition to revealing a general approach to investigating rare genetic diseases, we demonstrate the biochemical foundations of therapeutic strategies targeting GPX4.

Sedaghatian-type spondylometaphyseal dysplasia (SSMD), first described in 1980, was reported as a rare neonatal lethal disorder characterized by severe metaphyseal chondrodysplasia with limb shortening, cardiorespiratory defects and central nervous system abnormalities. Most infants with SSMD succumb within days after birth owing to respiratory distress. To date, four SSMD-related GPX4 variants have been described: c.476+5G>A, c.477-8_477-4del, c.270C>T (p.Tyr127*) and c.153_160del3,4. Considering the severe impact of these biallelic frameshifting or truncating mutations on GPX4 function, the clinical phenotypes of SSMD patients are probably due to complete loss of GPX4 enzymatic function, although the exact pathophysiological mechanism is unknown.

GPX4 is a selenoprotein and a member of the glutathione peroxidase family of enzymes, which share an antioxidant function of reducing peroxides through use of the cosubstrate glutathione. Despite structural and functional similarities, GPX4 is distinct from other GPX enzymes, being the only enzyme in mammals capable of reducing esterified phospholipid hydroperoxides and cholesterol hydroperoxides within the context of cell membranes. Therefore, when GPX4 activity is compromised, accumulation of lipid peroxidation products can disrupt membrane architecture, resulting in cell death through ferroptosis, an iron–dependent form of nonapoptotic cell death. Accordingly, an essential role for GPX4 during embryogenesis and early development has been suggested by the failure of GPX4 homozygous knockout mice to survive past early gestation.

Here, using whole-exome sequencing, we identified three individuals with mild SSMD features from two unrelated families who were found to harbor a recurrent homozygous point mutation in the GPX4 gene, c.455G>A (p. R152H). We sought to investigate the impact of this patient-derived GPX4 missense variant on the function of GPX4 protein, to guide SSMD patients towards treatments. Furthermore, structural examination of the variant suggested that K48 has an essential role in modulating GPX4 function, in addition to the previously reported catalytic triad (Sec46/Gln81/Trp136) and Asn137 (ref. 9). We further found that the R152H variant alters the degradation of GPX4, revealing the degradation mechanism of GPX4 protein.

Results

R152H variant causes substantial loss of function. A recurrent homozygous R152H point mutation in the GPX4 gene was identified in the whole-exome sequencing of three patients with SSMD features (Supplementary Table 1). To investigate the impact of the R152H variant on GPX4 structure and function, we began with computational modeling of the GPX4R152H protein structure. Substitution of Arg152 by His in the crystal structure of GPX4 was followed by global minimization of the structure in an implicit solvent to generate a GPX4R152H structural model.

Comparing GPX4R152H with GPX4WT, we found that the R152H variant substantially altered the surface around residue 152, as evidenced by the loss of a hydrophobic pocket centered on Arg152 in the wild-type (WT) protein (Fig. 1a). The change in the surface features mainly derived from a conformational change in the loop between Pro124 and Ala133, with which the long side chains of Arg152 formed multiple hydrogen bonds in the WT protein to...
support the loop in an open conformation, but not in the His variant, which has a short side chain and fewer H-bond donors such that the loop collapsed (Fig. 1b).

Molecular dynamics (MD) simulations of GPX4R152H and GPX4WT predicted this loop to be exceptionally mobile in the context of the variant (Fig. 1c). GPX4R152H also exhibits additional flex-
ability in its active site (Supplementary Videos 1 and 2). Accordingly, the average distance between the active site catalytic residue Sec46 and its catalytic partners Gln81/Trp136 was substantially increased in GPX4R152H and showed a considerably wide distribution over the timescale of the dynamics simulated, indicating a predicted weaker interaction among the catalytic triad (Fig. 1d).

As a control, GPX4R152H was also generated by synonymous mutation of Arg152 to Arg using the same method. Substantial structural alterations caused by the R152H mutation were observed in a comparison of GPX4R152H with GPX4U46C, excluding artificial effects associated with the analysis (Extended Data Figs. 1 and 2a,b, and Supplementary Videos 3 and 4). In addition, the presence of water in the MD simulation box considerably increased the mobility of the G126–T139 loop in GPX4U46C compared with water-free simulations, suggesting that water alone is not enough to restore hydrogen bonds to stabilize this loop (Supplementary Table 2 and Supplementary Videos 5 and 6).

To experimentally determine the impact of this variant in a human cell context, we established a cell model of the R152H mutation by stably overexpressing either green fluorescent protein (GFP)-tagged GPX4WT or GFP-tagged GPX4R152H in HT-1080 fibrosarcoma cells, in which GPX4 functions to protect cells from ferroptosis (Extended Data Fig. 3a). Using HT-1080 cells transfected with empty vector as a control, we measured the enzymatic activity of the transfected GFP–GPX4 protein via its ability to reduce a phospholipid hydroperoxide in an NADPH (reduced nicotinamide adenine dinucleotide phosphate)-coupled assay, as reported previously10. By normalizing enzyme activity to protein level, as measured by western blotting, we found that one unit of GPX4R152H exhibited ~40% of the activity of GPX4WT in this scenario (Fig. 2a and Extended Data Fig. 3b,c). To further confirm the variant-induced loss of activity in different genetic backgrounds and exclude interference from endogenous WT GPX4, using GPx4-knockout HT-1080 human cells and Pfa1 murine cells, we overexpressed human or murine, WT or R152H exogenous GPX4 (refs. 51–53). We found that GPx4-knockout cells expressing GPX4U46C, similar to GPx4-knockout cells, require α-tocopherol for normal growth, as they started to exhibit lower viability on day 7 after removal of α-tocopherol from the cell culture media, which is not the case for cells expressing GPX4WT; this suggests a profound impact of the R152H variant on GPX4 function (Fig. 2b). To characterize this effect further, we measured the GPX4 enzymatic activity of engineered Gpx4-knockout cells cultured in medium with or without α-tocopherol and found an increasing loss of activity over time for R152H GPX4 without α-tocopherol (Fig. 2c and Extended Data Fig. 3d). Because the observed gradual loss of activity specifically for R152H GPX4 is correlated with the cell death timeline and is in advance of the decrease in viability, it suggests that the accumulation of lipid peroxides caused by the less active R152H GPX4 will further deactivate R152H GPX4 and that incremental loss of activity eventually leads to compromised cell viability.

We therefore predicted that the characteristic reduction in enzyme activity in R152H GPX4 would result in impaired resistance to ferroptosis. Accordingly, we tested the ferroptosis sensitivity of HT-1080 cells overexpressing comparable levels of GFP–GPX4WT or GFP–GPX4R152H (Extended Data Fig. 4a). Although both cell lines were more resistant to ferroptosis induced by the GPX4 inhibitors RSL3 and ML162 and by the system x− inhibitor imidazole ketone erastin (IKE), compared with a cell line transfected with empty vector, overexpression of GPX4R152H was less protective against ferroptosis inducers than was GPX4WT, consistent with the lower activity of this variant protein for reducing lipid hydroperoxides (Fig. 2d).

These data suggest that the partial loss of GPX4 phospholipid peroxidase activity caused by R152H alteration probably contributes to the overall pathological phenotype in SSMD patients harboring this variant. Additionally, because a relatively low level of cellular GPX4 activity provided by a small amount of WT GPX4 was reported to be sufficient for cell survival in some contexts, R152H-specific vulnerability to deactivation by accumulation of lipid peroxide indicates a sensitivity of R152H GPX4 to overoxidation and therefore differentiates the lower activity of R152H GPX4 from equivalent activity provided by the WT GPX4 (ref. 13). Beyond the observed activity loss, considering the profound impact of R152H on GPX4, we suspect that R152H may also impact GPX4 function through additional mechanisms.

R152H causes substantial conformational change. To further understand why the alteration of a single amino acid residue distant from the active site caused a significant loss of enzymatic function in GPX4, we solved the X-ray crystal structure of GPX4U46C and GPX4R152H at 1.5 Å resolution (Extended Data Fig. 4b,c). The backbone of GPX4R152H aligned well with GPX4U46C, and the most outstanding change was in the loop between Pro124 and Ala133, which was intrinsically disordered in GPX4R152H, as evidenced by the loss of electron density corresponding to these residues (Fig. 2e,f). As modeling has suggested, this change is probably due to the loss of multiple hydrogen bonds that Arg152 forms with the backbone carbonyls of Gly126, Asn132 and Ala133, such that the loop becomes exceptionally flexible when this arginine is not present. This change is consistent with our observation that GPX4R152H exhibited a lower melting temperature than GPX4U46C (ΔTm, = −4 °C), which suggested R152H resulted in a conformational change in the GPX4 structure and substantially decreased the thermal stability of GPX4 protein (Fig. 2g).

Moreover, because active site residue Trp136 is in this disordered loop, we examined the active site and found distances between pairs of the catalytic residues were increased, as expected from modeling.
Although this observation may partly explain the decrease in catalytic activity, we also noticed in the X-ray structure an outstanding shift of the side chain of Lys48 away from the active site (Extended Data Fig. 4f). The exceptional mobility of Lys48 was also predicted by modeling. Because the positively charged Lys48 features strong interactions with the active site selenium/sulfur anion in the structure of GPX4 WT and our MD simulation of GPX4 U46C, we reasoned that Lys48 might have an important role in the enzymatic function of GPX4, and its departure from the active site may impair enzymatic activity (Supplementary Video 7). 

Lys48 has an essential role in modulating GPX4 activity. To further investigate the role of Lys48 in the enzymatic function of GPX4, we generated HT-1080 cells stably overexpressing an alanine mutant of GPX4, GFP–GPX4 K48A or GFP–GPX4 K48L, in which the mutation removed the positively charged ε-amino group on the side chain. Strikingly, we found that GPX4 K48A had an almost complete loss of activity to reduce phospholipid hydroperoxides in this context, as its overexpression did not effectively increase overall GPX4 activity in HT-1080 cells, which is in contrast to the overexpression of WT GPX4 (Fig. 3a and Extended Data Fig. 5a). By contrast, we found

**Extended Data Fig. 4**

- **Extended Data Fig. 4a**: Graph showing normalized activity of WT and R152H in cell viability experiments. 
  - **WT**: 1.0, **R152H**: 0.6 
  - **Supplemented with α-tocopherol**: 1.0, **Day 7 without α-tocopherol**: 0.5

- **Extended Data Fig. 4b**: Graph showing cell viability of GPX4 mutants in HT-1080 cells. 
  - **WT**: 1.0, **R152H**: 0.5 
  - **Supplemented with α-tocopherol**: 1.0, **Day 7 without α-tocopherol**: 0.5

- **Extended Data Fig. 4c**: Graph showing normalized GPX4 activity in HT-1080 cells. 
  - **WT**: 1.0, **R152H**: 0.5 
  - **Supplemented with α-tocopherol**: 1.0, **Day 7 without α-tocopherol**: 0.5

- **Extended Data Fig. 4d**: Graph showing cell viability of RSL3 and ML162 treatments. 
  - **WT**: 1.0, **R152H**: 0.5 
  - **Supplemented with α-tocopherol**: 1.0, **Day 7 without α-tocopherol**: 0.5

- **Extended Data Fig. 4e**: Graph showing normalized activity of GPX4 mutants in HT-1080 cells. 
  - **WT**: 1.0, **R152H**: 0.5 
  - **Supplemented with α-tocopherol**: 1.0, **Day 7 without α-tocopherol**: 0.5

- **Extended Data Fig. 4f**: Graph showing derivative reporter (ΔTm) of GPX4 mutants. 
  - **WT**: -2 × 10^3, **R152H**: -3 × 10^3 
  - **Supplemented with α-tocopherol**: -2 × 10^3, **Day 7 without α-tocopherol**: -3 × 10^3
that GPX4K48L featured enhanced enzymatic activity, in line with a previous observation that the recombinant K48L mutant of GPX4 had higher catalytic activity than WT14.

Correspondingly, although overexpression of GFP–GPX4K48A in HT-1080 cells did not show further protective effects in addition to endogenous GPX4 against ferroptosis induced by RSL3, overexpression of GFP–GPX4K48L was more protective than WT GPX4 against RSL3 (Fig. 3b and Extended Data Fig. 5b). These results suggested a profound and intriguing modulatory role of Lys48 on GPX4 activity.

Fig. 3 | Lys48 modulates the enzymatic function of GPX4. 

a, Using HT-1080 transfected with pBP empty vector as a control, the activities of WT, K48A and K48L GFP-tagged cytosolic GPX4 were measured in an NADPH-coupled GPX4 activity assay. Data are plotted as means ± s.d. of 11 replicate experiments. Ordinary one-way ANOVA followed by Tukey’s multiple comparisons test was performed (n = 11, d.f. = 30). P values were plotted. 
b, Viabilities of HT1080 overexpressing exogenous WT, K48A or K48L GFP–GPX4 and a control line were measured after treatment with RSL3 (62.5 nM) for 48 h. Data are plotted as means ± s.d. (n = 3 biologically independent samples). Two-tailed t-tests were performed and P values plotted. 
c, The crystal structure of GPX4U46C_K48A aligned with that of GPX4U46C. 
d, The crystal structure of GPX4U46C_K48L aligned with that of GPX4U46C. 
e, The distances between catalytic residues Cys46 and Trp136 were recorded every 4.8 ps for ten independent 100-ns MD simulations of GPX4U46C, GPX4U46C_K48A and GPX4U46C_K48L. All n = 208,350 independent measurements of distances throughout the ten independent simulations were plotted, with means ± s.d. An unpaired two-tailed t-test was then performed and P values plotted. 
f, In the crystal structure of oxidized GPX4U46C (Cys46–SO3H), Lys48 is in close proximity to the oxidized Cys46. The Fo–Fc omit map (cyan mesh) contoured at 3σ. The side chain of E65 (cyan) belongs to protomer B. The solid red sphere represents a water molecule in the active site. 
g, The distances between catalytic residues Cys46 and Trp136 were recorded every 4.8 ps for five 100-ns MD simulations of oxidized GPX4U46C, GPX4U46C_K48A and GPX4U46C_K48L (Cys46–SO3H). All n = 20,835 independent measurements of distances over the simulation time were plotted, with means ± s.d. An unpaired two-tailed t-test was performed and P values plotted. 
h, Viabilities of HT1080 overexpressing exogenous WT, K48A or K48L GFP–GPX4 and a control line were measured after treatment with IKE (0.6 µM) for 48 h. Data are plotted as means ± s.d. (n = 3 biologically independent samples). Two-tailed t-tests were performed and P values plotted.
To shed light on the mechanism for this essential modulation, we solved the crystal structures of GPX4\(^{46C,K48A}\) and GPX4\(^{46C,K48L}\) protein (Extended Data Fig. 5c,d). Although the GPX4\(^{46C,K48A}\) and GPX4\(^{46C,K48L}\) proteins are stable and superimpose well upon the structure of GPX4\(^{46C,K48A}\) (Fig. 3c,d), MD simulations of these three structures revealed additional flexibility in the active site of GPX4\(^{46C,K48A}\), whereas enhanced stability was observed in the active site of GPX4\(^{46C,K48L}\) (Supplementary Videos 7–9). Accordingly, the average distance between the active site catalytic residue Cys46 and its catalytic partner Trp136 was significantly increased in GPX4\(^{46C,K48A}\), which indicated a weaker interaction, whereas the average distance in GPX4\(^{46C,K48L}\) was even lower than in GPX4\(^{46C}\), which indicated a stronger interaction (Fig. 3e). Consistent results were also observed in MD simulations of computationally modeled Lys48 variants of WT selenocysteine-containing GPX4 (Extended Data Fig. 5e and Supplementary Videos 10–12). This suggests that the interaction between Lys48 and (seleno-)Cys46 modulates the activity of GPX4. The highest covalent-docking affinities were obtained with GPX4\(^{46C,K48L}\) and GPX4U46C\(_{K48L}\), and found that oxidized GPX4\(^{46C}\) exhibited additional flexibility in the active site, which suggests that the oxidized active site is in an open state. However, oxidized GPX4\(^{46C,K48A}\) and GPX4\(^{46C,K48L}\) featured extreme stability (Fig. 3g and Supplementary Videos 13–15). Because the SeO\(^{-}\)S\(^{-}\) state of GPX4 is inactive and irreversibly overoxidized, especially for the sulfur variant\(^{14}\), the stability of the K48A mutant in this state might contribute to its loss of activity.

The second step in the catalytic cycle of GPX4 is the incorporation of its cofactor reduced glutathione (GSH) via formation of an Se–S bond, which prepares oxidized GPX4 for regeneration to the reduced form. Therefore, we covalently docked GSH into the crystal structures of fully oxidized GPX4\(^{46C}\), GPX4\(^{46C,K48A}\) and GPX4\(^{46C,K48L}\), and found that oxidized GPX4\(^{46C}\) exhibited additional flexibility in the active site, which suggests that the oxidized active site is in an open state. In addition, the flexible and open active site of oxidized GPX4\(^{46C}\), as found during its MD simulation, might also be more accessible for GSH to incorporate and reduce oxidized GPX4.

The above analysis suggested an elevated susceptibility of K48L to overoxidation, in spite of its higher initial activity, because loss of the positively charged \(\varepsilon\)-amino group in the K48L mutant might have an impact on its interaction with the negatively charged oxidized active site or the GSH cofactor. To validate our analysis, we tested the sensitivity of HT1080 cells overexpressing comparable levels of GFP–GPX4\(^{46C}\) or GFP–GPX4\(^{46C,K48L}\) with ferroptosis induced by system \(\chi_{\text{c}}\) inhibitor IKe, which depleted cellular GSH and may therefore cause overoxidation of GPX4. We found that overexpression of GFP–GPX4\(^{46C}\) in HT1080 cells did not show further protective effects in addition to the endogenous GPX4 against IKe (Fig. 3h and Extended Data Fig. 6b).

In addition to K48A and K48L, we computationally modeled additional Lys48 variants of WT GPX4 and performed the above analysis on K48E (negative charge), K48Q (hydrogen bond) and K48R (positive charge and hydrogen bond). The broad performance spectrum observed with these Lys48 variants further supported the profound modulatory role of Lys48 in GPX4 activity (Extended Data Fig. 5e, Supplementary Table 3 and Supplementary Videos 16–18).

In summary, these data reveal a multipronged mechanism for the modulation of GPX4 enzymatic activity by Lys48: to stabilize the active site in a more compact and functional state, to modulate the oxidized active site for cycling and to facilitate incorporation of the cofactor GSH. Therefore, the shift in Lys48 away from the active site in GPX4\(^{46C,K48L}\) may contribute to the partial loss of function and specific sensitivity to overoxidation observed with GPX4\(^{46C,K48L}\).

**R152H variant is resistant to degradation.** When we tested the ferroptosis sensitivities of HT1080 cells overexpressing GFP–GPX4\(^{R152H}\) or GFP–GPX4\(^{WT}\), we were surprised to find that GPX4\(^{R152H}\) provided protection comparable with GPX4\(^{WT}\) to the ferroptosis inducer FIN56, despite insufficient protection against RSL3, ML162 and IKE (Fig. 4a and Extended Data Fig. 7a). Because FIN56 was reported to induce ferroptosis by promoting degradation of GPX4, as well as depleting coenzyme Q10 (CoQ\(_{10}\)) we speculated that R152H may change the susceptibility of GPX4 to degradation\(^{15}\). Therefore, we treated HT-1080 cells overexpressing GFP–GPX4\(^{R152H}\) or GFP–GPX4\(^{WT}\) with various ferroptosis inducers and measured the residual GPX4 protein after treatment by western blotting, using methods reported previously (Extended Data Fig. 7b)\(^{16}\). The endogenous GPX4 protein served as internal control and confirmed that RSL3, ML162 and FIN56 substantially promoted degradation of GPX4 under these treatment conditions (Fig. 4b and Extended Data Fig. 7c,d). Meanwhile, substantial degradation of transfected GFP–GPX4\(^{R152H}\) was induced by RSL3, ML162 and FIN56, as expected, which excluded interference of the GFP tag on the degradation (Fig. 4c and Extended Data Fig. 7e). We observed that GFP–GPX4\(^{R152H}\) was more resistant than GFP–GPX4\(^{WT}\) to degradation induced by RSL3, ML162 and FIN56 (Fig. 4d and Extended Data Fig. 7f). We further conducted cycloheximide-chase analyses of WT and R152H GFP–GPX4 under RSL3 treatment, which verified the increased half-life of R152H GPX4 under this condition (Fig. 4e and Extended Data Fig. 8a).

The mechanism of GPX4 degradation induced by RSL3 and FIN56 is not clear, despite efforts to understand this phenomenon\(^{12,13}\). Based on our finding that the loop between Pro124 and Ala133, which contains Lys125 and Lys127, exhibits extra flexibility in the context of GPX4\(^{R152H}\), we developed a hypothesis that RSL3- or FIN56-induced GPX4 degradation involves a proteasome-dependent mechanism, with Lys125 and Lys127 as potential ubiquitination sites on GPX4; in this model, these highly mobile lysine residues in R152H hinder ubiquitination and prevent degradation. We tested this hypothesis by examining GPX4 degradation in HT1080 cells stably overexpressing GFP–GPX4\(^{R125SR,K127R}\), in which the proposed sites of ubiquitination were removed by point mutations. Whereas endogenous GPX4 was degraded upon treatment with RSL3, ML162 or FIN56, we found that GFP–GPX4\(^{R125SR,K127R}\) was resistant to degradation, even more so than the R152H mutant (Fig. 4f and Extended Data Fig. 8b,c). Together, these data suggest that degradation of GPX4 induced by RSL3 and FIN56 involves a proteasome-dependent mechanism, and that Lys125 and Lys127 of GPX4 are key sites of ubiquitination. An alternative approach to modulate GPX4 for clinical applications was thus revealed.

**Impact of R152H variant is similar in patient fibroblasts.** To validate our observations regarding the R152H variant, fibroblasts developed from a patient with homozygous R152H variants...
(RAG01, GPX4R152H/R152H) and the patient’s parent as an unaffected control with heterozygous genotype (RAG02, GPX4R152H/WT) were tested. Although the two human fibroblast cell lines expressed an equivalent level of GPX4 protein based on western blot analysis, RAG01 cells exhibited significantly less GPX4 enzymatic activity than RAG02, confirming that R152H variant in GPX4 caused partial loss of enzymatic function (Fig. 5a,b). As expected, RAG01 with a partial loss of GPX4 activity was found to be more sensitive than RAG02 to lipid peroxidation and to ferroptosis induced by RSL3, ML162, FIN65 and IKE. Data are plotted as means with range of two biologically independent experiments. The corresponding blots are shown in Extended Data Fig. 4b,c.

Identification of proof-of-concept treatment. The above results demonstrated that the patient-derived R152H variant of GPX4 caused a partial loss of function in phospholipid peroxidase activity and therefore is pathological. However, the data also suggested that overexpression of the partially active but degradation-resistant GPX4R152H in HT-1080 cells did increase the resistance of cells to ferroptosis induced by lipid peroxidation, although less effectively than WT GPX4 (Fig. 2d). Therefore, we developed a hypothesis that boosting expression of the R152H variant of the selenoprotein GPX4 via selenium supplementation might compensate for the partial loss of enzymatic function. This is based on the observation that selenium supplementation can effectively increase expression of the selenoprotein GPX4, up to 48-fold, as well as expression of another antioxidant selenoprotein, GPX1, up to 40-fold, in human cells20. In

**Fig. 4** | Resistance of GPX4R152H to degradation induced by GPX4 inhibitor revealed the ubiquitin/proteosome-dependent mechanism of the GPX4 degradation induced by FIN56/RS3. **a**. Viabilities of HT-1080 cells overexpressing exogenous WT or R152H GFP–GPX4 and a control line were measured after treatment with FIN56 (0.6 μM) for 48 h. Data are plotted as means ± s.d. (n = 3 biologically independent samples). Two-tailed t-tests were then performed for comparison and P values plotted. **b**. Endogenous GPX4 in HT1080 cells overexpressing GFP–GPX4WT were tested for vulnerability to degradation induced by RSL3, ML162, FIN65 and IKE. Data are plotted as means with range of two biologically independent experiments. The corresponding blots are shown in Extended Data Fig. 4b,c. **c**. GFP–GPX4WT in HT1080 cells overexpressing GFP–GPX4WT was tested for vulnerability to degradation induced by RSL3, ML162, FIN65 and IKE. Data are plotted as means with range of two biologically independent experiments. The corresponding blots are shown in Extended Data Fig. 4d. **d**. GFP–GPX4R152H in HT1080 cells overexpressing GFP–GPX4R152H was tested for vulnerability to degradation induced by RSL3, ML162, FIN65 and IKE. Data are plotted as means with range of two biologically independent experiments. The corresponding blots are shown in Extended Data Fig. 4e. **e**. Cycloheximide-chase analysis of GFP–GPX4WT, GFP–GPX4R152H and endogenous WT GPX4. Means of two biologically independent experiments were plotted. The corresponding blots are shown in Extended Data Fig. 4f. **f**. GFP–GPX4K125R/K127R in HT1080 cells overexpressing GFP–GPX4K125R/K127R was tested for vulnerability to degradation induced by RSL3, ML162, FIN65 and IKE. Data are plotted as means with range of two biologically independent experiments. The corresponding blots are shown in Extended Data Fig. 4h. DMSO, dimethylsulfoxide; WB, western blotting.
addition, selenium is readily available from dietary source such as Brazil nuts and as an over-the-counter supplement. We first tested this selenium treatment hypothesis on fibroblasts derived from the patient. We found that sodium selenite, selenomethionine and methylselenocysteine could boost the viability of patient fibroblast RAG01 slightly to 138%, 138% and 137%, with an EC50 (half maximal effective concentration) value of 0.8, 0.4 and 0.9 nM, respectively (Fig. 6a and Extended Data Fig. 9a). The similarity in the increased viability for these three seleno compound treatments suggested a consistency of selenium supplementation, and their shared low EC50 values indicated their high potencies as treatments. Because the CC50 (50% cytotoxic concentration) of sodium selenite toxicity (12 µM) was 15,000-fold higher than its EC50 (3 µM), RAG01 and RAG02 were tested for GPX4 protein expression level by western blotting. Data are plotted as means ± s.d. (n = 3 biologically independent samples). An ordinary two-way ANOVA followed by Sidak’s multiple comparisons test was performed and plotted (t = 0.5155, d.f. = 58, P = 0.6081). f, Ratio of mean cytoplasm immunofluorescence of GPX4 to mean nuclear immunofluorescence of GPX4 observed in RAG01 and RAG02. Data are plotted as means ± s.d. (n = 30 biologically independent samples). An unpaired two-tailed t-test was performed and plotted (t = 1.846, d.f. = 58, P = 0.0713). g, Nine representative immunofluorescence images for RAG01 and RAG02 showing DAPI (blue) and GPX4 fluorescence (red). Scale bar, 20 µm. DMSO, dimethylsulfoxide.
selenomethione and methylselenocysteine might be preferred owing to their lack of toxicity. It was also noteworthy that the rescue effect of selenium was specific for RAG01, with relatively minimal effect on the control RAG02 line.

Moreover, we included N-acetylcysteine and N-acetylcysteine amide in the test as metabolic precursors for the GPX4 cofactor GSH, which might also benefit patients. We found that N-acetylcysteine and N-acetylcysteine amide could boost the viability of RAG01 cells slightly to 118% and 116%, which was less effective than selenocysteine (Fig. 6c and Extended Data Fig. 9b). This suggests the utility of selenium for patients with compromised GPX4 activity, and that elevation of GSH in cells might be helpful in boosting the activity of GPX4.

In addition, we evaluated the lipophilic antioxidants α-tocopherol, CoQ10, and idebenone (a soluble analog of CoQ10), and found that they can also boost the viability of RAG01 cells to 250%, 197% and 245%, with EC50 values of 115 nM, 5 μM and 228 nM, respectively (Fig. 6d–f). We also found that dimethyl fumarate, an Nrf2 (nuclear factor erythroid 2-related factor 2) activator that promotes an antioxidant response, boosted the viability of RAG01 slightly to 127% (Fig. 6g)21. These results suggest that lipophilic antioxidant supplements, and possibly dimethyl fumarate to a limited extent, may be useful for patients with the R152H GPX4 variant.

Finally, we found that treatment with deuterium-reinforced linoleic acid (RT-001), a polyunsaturated fatty acid with deuterium at its bis-allylic site to inhibit lipid peroxidation, had the most pronounced rescue effect on RAG01 (440%, Fig. 6h)23. By contrast, normal linoleic acid was toxic to RAG01 at high concentrations, which suggested the likely involvement of lipid peroxidation in the compromised cell viability observed on patient cells (Fig. 6i).

To further evaluate the potency of the proof-of-concept treatments and compare their effects on GPX4R152H with a control expressing WT GPX4 (instead of RAG02 expressing both wild-type and the variant), we tested all treatments on GPX4-knockout Pfa1 cells that were transfected to overexpress human/murine GPX4WT or GPX4R152H (Extended Data Figs. 9c and 10). Ferrostatin-1, a radical-trapping inhibitor of lipid peroxidation and ferroptosis, was also included in this test; its substantial and selective rescue effect on cells solely expressing GPX4R152H demonstrated the involvement of lipid peroxidation and possibly ferroptosis in R152H GPX4 pathology. Proof-of-concept treatments effective on patient fibroblasts also showed consistent rescue effects on the Pfa1 cells, especially for α-tocopherol, CoQ10, idebenone and RT-001.

In summary, the observation that selenium supplementation, ferroptosis inhibitors and antioxidants can increase the number and viability of patient fibroblasts and R152H GPX4 cell models were consistent with the conclusion that the partial loss-of-function R152H mutation in GPX4 sensitizes patient cells to lipid peroxidation. Therefore, we expect that a combination treatment of selenium supplementation to boost R152H GPX4 levels and lipophilic antioxidants (α-tocopherol and/or CoQ10) with deuterated polyunsaturated fatty acids such as RT-001, some of which are widely
available as supplements, to suppress lipid peroxidation might be the most effective treatment and may be therapeutically beneficial for patients with the R152H variant in GPX4.

Discussion

Before this study, patients with SSMD were identified as having predicted loss-of-function variants based on in silico data alone; the variants were not studied experimentally. Our report provided evidence of the association of biallelic variants in GPX4 with SSMD. In addition, this report extends the phenotype associated with SSMD to include long-term survival beyond infancy.

This study began by examining the effect of the R152H missense mutation on GPX4, which adversely changed the protein structure and caused a partial loss of antioxidant activity. We hypothesize that, as a hypomorphic allele, there is sufficient enzymatic function to allow for survival beyond infancy, but with impaired proliferation and increased susceptibility to lipid peroxidation, ferroptosis, tissue damage and degeneration. This is in keeping with observations of the adult conditional Gpx4-knockout mouse, which exhibits perinatal lethality, seizures, ataxia and progressive neuronal loss.

During our analysis, we used structure-based computational modeling to study the effect of the variant on protein structure in silico, predictions from which were confirmed by protein crystal structures and cellular assays. These experiments suggested the feasibility of structure-based modeling of variant protein structures to dissect the impact of patient-derived variants. The low cost and high throughput of computational modeling would potentially benefit more patients with orphan diseases or variation in key genes.

This R152H variant unexpectedly revealed the structural basis of GPX4's enzymatic activity and regulation of its degradation: Lys48 was found to modulate GPX4 enzymatic activity and Lys125/Lys127 were revealed as sites of ubiquitin ligation. In addition, our study of R152H suggested Arg152 as an allosteric site indirectly regulating GPX4 activity. Because recent studies highlighted GPX4 protein as an Achilles heel of drug-resistant and metastatic cancers, which are exceptionally dependent on the GPX4 lipid peroxide repair pathway, we would expect biochemical therapeutic strategies targeting the essential residue Lys48 and allosteric site Arg152, or taking advantage of its degradation mechanism, to be developed as a high priority and benefit additional patients.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgement, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41589-021-00915-2.

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In this study, we identified three individuals with SSMD features who were found to harbor a recurrent homozygous R152H point mutation in the GPX4 gene. At 5 months of age, patient 1 (family 1) was diagnosed on brain auditory-evoked responses testing to have neurosensorineural hearing loss. The metabolic bone clinic suggested metaphyseal dysplasia. Whole-exome sequencing of patient 1 and his parent was conducted. Only one gene variant was identified, c.455G>A (p. R152H) of GPX4, which was found to segregate in each parent and presented in the patient as a homozygous biallelic variant.

A skeletal survey of patient 2 (family 2) at 7 years of age revealed flaring of the proximal tibial metaphysis as well as distal femoral metaphysis, brachycephaly, moderate thoracolumbar lordosis, coxa valga, thin and elongated second metatarsals and diffuse osteopenia with thinning of the long bones. Patient 3 (family 2) is the younger sister of patient 2. Patient 3 did not pass her newborn hearing exam and second metatarsal and fibular variant responses were noted.

Whole-exome sequencing results are summarized in Supplementary Table 1. In the custom analysis tool Xome Analysis to detect the patient-derived variant. NextGen sequencing (massive parallel sequencing) was performed by the Agilent Center for Genomics Research at Columbia University. Compensation was not provided.

In silico calculation of the GPX4R152H crystal structure that we solved (PDB: 7L8L) was not used for MD simulation because the loop between P124 and A133 does not exhibit a defined structure, which would complicate the modeling. It is also noteworthy that a previously published crystal structure of GPX4U46C (PDB: 20B8) was used above to model the GPX4R152H structure. This is because this modeling was done before all other experiments in a wet lab, including acquisition of our own crystal structure of GPX4R152H (PDB: 7L8K).

To further study the impact of R152H mutation with MD simulation, each of the above modeled structures was set up in an orthorhombic box with 0.15 M NaCl in simple point-charge (SPC) water and an OPLS3e force field, unless noted otherwise. Multiple independent MD simulations of each system for 100 ns with 4.8 ps per step at 300 K and 1.01325 bar were performed with random seeds in the Trajektor Molecular Dynamics Trajectories were recorded and analyzed. Simulation quality and event analysis were also performed using Desmond. Videos of representative simulation processes were exported.

In this study, we identified three individuals with SSMD features who were physically purified in vitro (GPX4R152H, GPX4A46C and GPX4K48L) have the U46C mutation to aid protein expression, we started from computational modeling on GPX4U46C with the U46C mutation to either to directly compare the crystal structures for the GPX4 protein (GPX4U46C) and the computationally predicted GPX4R152H structure side-by-side, or to use our crystal structures of GPX4 protein (GPX4A46C and GPX4K48L) as starting points for MD simulations. We then performed computations on GPX4R152H (PDB: 6HN3) as a confirmatory control to rule out the possibility of artificial effects caused by the computation process. For models that featured the U46C mutation, the cysteine thiol of C46 was manually set to a thiol anion to mimic the active form of the enzyme because the selenocysteine has a lower pKₐ value and would convert to the anion form under physiological conditions. SiteMap was then run under the default setting. A structural comparison of GPX4R152H and GPX4R152H was performed with Salsa.

To study the impact of K48 mutations on reduced GPX4, the GPX4A46C (PDB: 7L8K), GPX4A46C_K48A (PDB: 7L8R) and GPX4A46C_K48L (PDB: 7L8M) crystal structures that we solved were respectively set up in an orthorhombic box and analyzed with MD simulations using the same protocol as noted above. Additional K48 variants modeled from GPX4R152H were also analyzed in the same protocol.

To study the impact of K48 mutations on oxidized GPX4, the fully oxidized GPX4A46C crystal structure (PDB: 7L8Q) that we solved was not used for MD simulation. Instead, for side-by-side comparison, we manually set the cysteine thiol of GPX4A46C (PDB: 7L8Q) to thiol anion (PDB: 7L8Q) before computing, and GPX4A46C_K125R_K127R (PDB: 7L8M) to be fully oxidized. After confirmation of the relevance of the modeled oxidized structure by comparing it with the crystal structure, we used these fully oxidized structures in the MD simulation, as described above.

In addition, covariant docking of GSH was performed on the crystal structures of GPX4A46C (PDB: 7L8K), GPX4A46C_K48A (PDB: 7L8R) and GPX4A46C_K48L (PDB: 7L8M), which were solved and reported in this work. Other docking receptors of GSH are the crystal structure of GPX4R152H (PDB: 6HN3) and modeled structures based on it.

In this study, we identified three individuals with SSMD features who were physically purified in vitro (GPX4R152H, GPX4A46C and GPX4K48L) have the U46C mutation to aid protein expression, we started from computational modeling on GPX4U46C with the U46C mutation to either to directly compare the crystal structures for the GPX4 protein (GPX4U46C) and the computationally predicted GPX4R152H structure side-by-side, or to use our crystal structures of GPX4 protein (GPX4A46C and GPX4K48L) as starting points for MD simulations. We then performed computations on GPX4R152H (PDB: 6HN3) as a confirmatory control to rule out the possibility of artificial effects caused by the computation process. For models that featured the U46C mutation, the cysteine thiol of C46 was manually set to a thiol anion to mimic the active form of the enzyme because the selenocysteine has a lower pKₐ value and would convert to the anion form under physiological conditions. SiteMap was then run under the default setting. A structural comparison of GPX4R152H and GPX4R152H was performed with Salsa.
kinetically at 1-min intervals over 20 min. GPX4 activity was calculated based on the decrease in the characteristic absorbance at 340 nm was monitored and quantified and the other part was used for the GPX4-specific activity assay.

For the GPX4 degradation study, cells were seeded at 800,000 per well in a 60-mm plate and allowed to adhere overnight. Cells were then treated with 100 µM α-tocopherol and either 1 µM RSL3, 1 µM ML162, 10 µM IKe, 10 µM FIN56 or vehicle for 10 h before harvest. Particularly for cycloheximide-chase analysis, cells were treated with 4 mM RSL3, 30 µM cytokine and 100 µM α-tocopherol for 0, 2, 4 or 6 h before harvest. Each condition was performed at least twice. Cells were harvested with trypsin (Invitrogen, catalog number 25200-114), pelleted and lysed with RIPA buffer.

For both experimental groups, western blotting of each condition or cell line was performed at least twice. Cell lysates were blotted and imaged as previously described. Antibodies used were GPX4 (Abcam, catalog number ab125066; 1:2500 dilution), GPX (Santa Cruz, catalog number sc-9996; 1:100 dilution), actin (Cell Signaling, catalog number D11C11; 1:5000 dilution), GAPDH (Santa Cruz, catalog number sc-47721; 1:1000 dilution), IRDye 800CW goat anti-rabbit IgG (LI-COR, catalog number 926-6802; 1:15,000 dilution) and goat anti-mouse IgG (H+L) secondary antibody conjugated with AlexaFluor 680 (ThermoFisher Scientific, catalog number A-21057; 1:5000). Results were quantified using LI-COR Odyssey CLX IR scanner and ImageJ, and plotted using GraphPad Prism.

Determination of GPX4-specific activity. We applied a NADPH-coupled cellular GPX4 enzymatic activity assay, as previously reported. Oxidized glutathione, generated by GPX4 during reduction of its specific phospholipid hydroperoxides substrate, was reduced by glutathione reductase at the expense of NADPH, and the decrease in the characteristic absorbance at 340 nm was monitored and quantified as GPX4 activity. The GPX4-specific substrate phosphatidylcholine hydroperoxide (POCOH) was prepared by enzymatic hydroperoxidation of phosphatidylcholine by soybean lipase type IV. 22 ml of 0.2 M Tris-HCl, pH 8.8, containing 3 mM sodium deoxycholate and 0.5 mM phosphatidylcholine was incubated at room temperature, under continuous stirring for 30 min with 0.7 mg of soybean lipase type IV. The mixture was loaded onto a Sep-Pak C18 cartridge (Waters-Millipore) washed with methanol and equilibrated with water. After washing with 10 volumes of water, phosphatidylcholine hydroperoxides were eluted in 2 ml of methanol.

To measure the GPX4 activity for specific cell lines of interest, 48 million cells per well of a 96-well plate on day 1. For cell lines that were also subjected to the GPX4-specific activity, the remaining cells (after seeding into plates) were immediately tested by western blotting and the activity assay described above. Compounds were dissolved in dimethylsulfoxide and a twofold dilution series was prepared. The compounds were then diluted 1:50 in media and 4 µl was added to each well of the plates on day 2. After 48 h of treatment, the viability of cells was measured using a 1:1 dilution of CellTiter-Glo luminescent reagent (Promega, catalog number G7573) with media, which was read on a Victor 5 plate reader after 10 min of incubation. The luminescence was normalized to that of the dimethylsulfoxide control. Results were quantified using GraphPad Prism v.9.

Protein purification. GPX4 is a selenoprotein with selenocysteine at its active site (U46). Large scale expression of this enzyme expressing a protein in recombinant systems is challenging because of inefficient selenocysteine-encoding machinery; thus, the selenocysteine to cysteine (U to C, inserting a thiol group in the catalytic tetrad and other structural properties discovered in the context of the U to C mutant, we used a GPX4U46C construct for in vitro structural studies, and simultaneously examined the selenocysteine-containing cytosolic GPX4 in structure-based computational analysis and human cells via enzymatic assays and cellular assays.

Bacterial expression vector pPO30-His-tagged-c-GPX4U46C has been described previously. With the vector as a template, the following mutagenesis primers were designed using the Agilent QuikChange Primer Design application: R512H (forward: 5’-CTGGTGGTTGAAAGCAGGTAGCACCATG3’; reverse: 5’-ACATGGTGCTGGCAACACTGACTGACG3’), K48L (forward: 5’-GAGTGTAGTTTACTTCGGTTAGGCCACACTGGGAGGCCACG3’; reverse: 5’-CGTGGCCTCCCAGTGTGGCCTAACCGAAGTAAACTACACTC-3’). Primers were purchased from Integrated DNA Technologies. A site-directed mutagenesis kit (QuikChange II, Agilent, catalog number 200521) was then used to acquire pOE30-c-GPX4U46C, pOE30-c-GPX4MC, pOE30-c-GPX4MC+K48L, and pOE30-c-GPX4MC, K48L. All mutations and the resulting plasmids were confirmed by sequencing at GENEWIZ.

Isolated colonies each with plasmid were separately transferred to 8 ml of Luria–Bertani (LB) medium with 100 µg ml−1 ampicillin, and the inoculated culture was incubated with shaking (225 r.p.m.) at 37 °C for 16 h. Three milliliters of starter culture was added to 1 l of fresh LB medium with 100 µg ml−1 ampicillin. The culture was incubated with shaking (225 r.p.m.) at 37 °C until the optical density at 600 nm reached 0.9. The temperature was then decreased to 15 °C. Cells were inoculated overnight with 1 mM isopropyl-β-D-thiogalactoside at 15 °C while being shaken (225 r.p.m.) for 5 h. The next day r.p.m. was increased to 300 r.p.m. 30 h later, the bacteria were harvested by centrifugation at 4000g for 20 min at 4 °C and the pellet obtained was used for purification or stored at −20 °C. The pellet was resuspended in 25 ml of chilled lysis buffer (100 mM Tris, pH 8.0, 300 mM NaCl, 20 mM imidazole, 3 mM tris(2-carboxyethyl) phosphate (TCEP) and Roche protease inhibitor cocktail). Bacteria were lysed by sonication on ice for 6 min, and the lysate was centrifuged at 10,000 r.p.m. for 20 min at 4 °C to remove cell debris. The clarified lysate was incubated with Ni Sepharose 6 Fast Flow beads (GE Life Sciences) on a rotating at 4 °C for at least 1 h. The beads were washed with wash buffer (100 mM Tris, pH 8.0, 300 mM NaCl, 50 mM imidazole and 3 mM TCEP) to remove nonspecific binding. The protein was eluted with 100 mM Tris, pH 8.0, 300 mM NaCl, 200 mM imidazole, 3 mM tris(2-carboxymethyl) phosphate (TCEP) and Roche protease inhibitor cocktail. The protein was further purified using a gel filtration Superdex 200 column in fast protein liquid chromatography (FPLC) buffer containing 100 mM Tris, pH 8.0, 300 mM NaCl and 3 mM TCEP. Fractions containing GPX4 were pooled and analyzed by SDS–polyacrylamide gel electrophoresis.

Crystallization and structure determination. Protein samples of GPX4U46C were initially screened at the High-Throughput Crystallization Screening Center of the Hauptman-Woodward Medical Research Institute (HWI) (https://hwibuffalo.edu/high-throughput-crystallization-center/). The most promising crystal hits were reproduced using an under oil micro batch method in a COY anaerobic glove box at 23 °C. Plate-like crystals of GPX4U46C were grown using a crystallization reagent comprising 0.056 M sodium phosphate monobasic monohydrate, pH 8.2, and 1.344 M potassium phosphate with a protein to crystallization reagent ratio of 2:1. All crystals were subsequently transferred into a similar crystallization reagent supplemented with 20% (v/v) glycerol and flash-frozen in liquid nitrogen in the glove box. A native dataset was collected on a crystal of GPX4U46C at the
NE-CAT24-ID-C beam line of Advanced Photon Source in Lemont, IL, USA. Crystals of GPX4-U46C were subsequently used as seeds for growing crystals of GPX4-U46C_R152H, GPX4-U46C_K48L, GPX4-U46C_K48A and GPX4-U46C-sulfone outside the glove box, albeit different crystallization conditions were used for growing the crystals. Crystals of GPX4-U46C-R152H were grown under crystallization conditions comprising 0.2 M sodium thiocyanate, pH 6.9, and 20% (v/v) polyethylene glycol (PEG) 8000, whereas crystals of K48L and K48A were grown under conditions of 0.1 M sodium chloride, 0.1 M MES, pH 6.4, and 40% (v/v) PEG 8000. Crystals of the fully oxidized GPX4-U46C-sulfone were grown in 0.1 M potassium thiocyanate, 0.1 M sodium acetate, pH 5, and 20% (v/v) PEG 8000, and were harvested after one month. In each case, crystals were transferred into the respective crystallization reagent, which was supplemented by 20% (v/v) ethylene glycol.

Crystals of GPX4-U46C, GPX4-U46C-R152H, GPX4-U46C-K48L, GPX4-U46C-K48A and GPX4-U46C-sulfone diffracted X-rays at a NE-CAT24-ID-C beam line to a resolution of 1.38, 1.61, 1.52 Å, respectively. The images were processed and scaled in space group $P$2_12_12_1 and $P$2_1, respectively. The structure of each protein was determined by molecular replacement using the MOLREPE1 program and the crystal structure of GPX4-U46C (PDB: 2OBI) was used as a search model for structure determination of GPX4-U46C. The structure was used as the search model for subsequent structure determination of other crystal structures. The geometry of each crystal structure was fixed using programs XtalView34 and COOT41, and refined by Phenix44. There is one protomer of GPX4 in the asymmetric unit of the crystal with space group $P$2_1, whereas the asymmetric unit of GPX4-U46C-sulfone with space group $P$2_12_12_1 contains two protomers. The crystallographic statistics is shown in Supplementary Table 4.

Thermal shift assay (determination of protein melt temperature). Because mutation of amino acid residues may alter the thermostability of a protein, we used a thermal shift assay to monitor the potential change in GPX4 thermostability caused by R152H mutation, which practically determined the change in the protein’s thermostability. Because of the small size of the protein, a thermal shift assay was performed on the ViiA 7 Real-Time PCR system (ThermoFisher Scientific) with the thermal protocol: 25°C for 15 s, increase temperature to 99°C at a rate of 0.05°C s$^{-1}$, 99°C for 15 s. Fluorescence was recorded and analyzed using Protein Thermal Shift software.

Immunofluorescence study and quantification. Human fibroblasts RAG01 and RAG02 were separately seeded on polylysine-coated coverslips (Sigma Aldrich, catalog number P4832) in a 24-well plate (100,000 cells per coverslip and three coverslips for each cell line) and allowed to grow overnight. Medium was removed and the cells were gently washed twice with PBS+ (PBS with 1 mM CaCl$_2$, and 0.05 mM MgCl$_2$). The cells were fixed and permeabilized by adding 200 µl per well of 4% paraformaldehyde in PBS buffer with 0.1% Triton X-100 (PBS-T), and incubated at room temperature for 20 min. The cells were washed three times with PBS-T, blocked with 5% goat serum (ThermoFisher Scientific, catalog number A-20361) and incubated at room temperature for 1 h. The cells were then incubated with monoclonal mouse GPX4 antibody (Santa Cruz, catalog number sc-166650; 1:500 dilution) in PBS-T with 1% BSA and 5% goat serum overnight at 4°C, followed by washing three times with PBS-T for 5 min each time. The cells were incubated with goat anti-mouse IgG (H+L) highly cross-adsorbed secondary antibody, AlexaFluor 594 (ThermoFisher Scientific, catalog number A-11032, RRID:AB_2354091; 1:200 dilution) at room temperature for 1 h and then washed with three times with PBS-T for 5 min each time. ProLong Diamond anti-fade mountant with DAPI (ThermoFisher Scientific, catalog number P36926) was added to stain the nucleus. All images were captured on a Zeiss LSM 800 confocal microscope at Plan-Apochromat x63/1.4 oil differential interference contrast objective with constant laser intensity for all samples.

Quantification of the intensity of antibodies was analyzed using CellProfiler v3.1.8 (ref. 10). (CellProfiler Image Analysis Software, RRID:SCR_007358). Nuclei were first identified as primary objects using a global minimum cross entropy strategy, based on the DAPI fluorescence signal. Whole cells were then identified as secondary objects based on the primary objects by propagation using a global minimum cross entropy strategy, based on the GPX4 fluorescence (AlexaFluor 594) signal. The cytoplasm was identified as the tertiary object as part of each cell excluding the nucleus. Mean intensities of GPX4 fluorescence of nuclei, cytoplasm and whole cells were measured and reported. Graphs were created in GraphPad Prism v9.

Statistical analyses. All experiments, unless otherwise indicated, are biological replicates based on distinct samples. All statistical analyses were performed using GraphPad Prism v9. Tukey’s test was performed as a post-hoc test after ordinary one-way ANOVA, comparing all pairwise datasets, with alpha = 0.05. Sidák’s test was performed as a post-hoc test after ordinary two-way ANOVA for grouped data, comparing pairwise datasets in each group. Only relevant pairwise comparisons are highlighted in the figures. Unpaired Student’s t-test was performed when indicated in the manuscript, for comparing two experimental conditions. All t-tests were two-tailed with a significance threshold of $P < 0.05$.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Crystal structural coordinates were deposited in the RCSB, with accession codes PDB IDs: 7L8K, 7L8L, 7L8M, 7L8R, and 7L8Q. Publicly available datasets used in this study are: PDB IDs: 2OBI, 6HN3. Source data are provided with this paper.

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**Author contributions**

B.R.S. conceived and implemented the project after discussions with R.S. The planning and design of experiments was performed by H.L., T.S., R.S., M.C. and B.R.S. Computational modeling was conducted by H.L. as were biophysical assays, biochemical assays and cellular experiments. H.L. and X.X. conducted protein purification. E.F. crystallized the proteins and collected diffraction data to solve the crystal structures. T.S. and M.C. prepared GPX4-knockout Pfa1 and HT1080 cells. R.S., K.W. and J.F. conducted clinical observations of the patients. M.S.S. provided deuterium-reinforced linoleic acid. H.L. and B.R.S. wrote the manuscript with input from all authors.
Competing interests
B.R.S. is an inventor on patents and patent applications related to GPX4 and ferroptosis, and is a consultant to and cofounder of Inzen Therapeutics and Nevrox Limited, and is a member of the Scientific Advisory Board of Weatherwax Biotechnologies Corporation, and a consultant to Akin Gump Strauss Hauer & Feld LLP. M.C. is an inventor of ferroptosis-related patents and cofounder and shareholder of ROSCUE Therapeutics GmbH. J.F. participates in clinical trials with Biogen (Angelman’s syndrome) and J.F.’s spouse is Founder and President of Friedman Bioventure, which holds a variety of publicly traded and private biotechnology interests. M.S.S. is the Chief Scientific Officer of Retrotrope, Inc.

Additional information
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Correspondence and requests for materials should be addressed to Brent R. Stockwell.
Peer review information Nature Chemical Biology thanks Dohoon Kim and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.
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Extended Data Fig. 1 | *In silico* analysis of the impact of R152H mutation on GPX4 (GPX4<sup>U46C</sup> PDB: 2OBI) structure. 

**a.** Structure of GPX4<sup>R152H</sup> was computationally modeled based on the crystal structure of GPX4<sup>U46C</sup> (PDB: 2OBI). As a control, structure of GPX4<sup>R152R</sup> was also computationally modeled using the same algorithm, which indeed represents WT GPX4 but excludes artifacts from computational process when compared with the modeled R152H protein structure. Technically the two modeled proteins are GPX4<sup>U46C-R152H</sup> and GPX4<sup>U46C</sup>. Protein surface are colored as below: hydrophobic (white), positive charge (red), and negative charge (blue). To visualize the pocket on top of R152 in the GPX4<sup>R152R</sup> structure, white dots were shown as indicator of space. Overlap of the R152H variant backbone with wild-type was performed in the panel on the right, where the major conformational change in the loop around His152 was colored (WT as in pale pink and R152H as in red). See Supplementary Note for rationale of using U46C GPX4.

**b.** The alternation of surface mainly derived from an outstanding conformational change of the loop between Pro124 and Ala133, with which the side chains of Arg152 formed multiple hydrogen bonds in the wild-type, but not His152 in the mutant.
Extended Data Fig. 2 | Molecular Dynamic (MD) simulation analysis of the impact of R152H mutation on GPX4. a, RMSF of each residue in MD simulation based on the modeled GPX4 structure. Representative data from 5 times 100 ns trajectories were plotted. b, Distances between Cys46 and its catalytic partners Gln81/Trp136 were monitored in the MD simulation of GPX4<sup>R152H</sup>, as compared to GPX4<sup>R152R</sup>. Representative data from 5 times 100 ns trajectories were plotted.
Extended Data Fig. 3 | Preparation of cell models of GPX4\(^{R152H}\). a, HT-1080 transfected with pBP GFP-cGPX4\(^{WT}\), pBP GFP-cGPX4\(^{R152H}\), pBabepuro (pBP) empty vector, pBP GFP-cGPX4\(^{K48A}\), pBP GFP-cGPX4\(^{K48L}\), and pBP GFP-cGPX4\(^{K125R-K127R}\) were selected with puromycin and imaged with microscope. Triplicate experiments were repeated independently with similar results while the representative images were shown. The plotted scale bar is 400 \(\mu\)m. b, Total GPX4 enzymatic activity (endogenous apo-GPX4 and transfected exogenous GFP-tagged-GPX4) of control HT1080 (pBP, no expression of GFP-tagged-GPX4) and HT1080 cells overexpressing GFP-GPX4WT or GFP-GPX4R152H. Data are plotted as means ± SD of six replicate experiments. Ordinary one-way ANOVA followed by Tukey’s multiple comparisons test was performed: n = 6, DF = 15 and P values were plotted. c, Western blot of control HT1080 (pBP) and HT1080 cells overexpressing GFP-GPX4\(^{WT}\) or GFP-GPX4\(^{R152H}\) using GPX4 and GAPDH antibodies. Expression levels of GFP-WT-GPX4 and GFP-R152H-GPX4 were quantified. Data are plotted as means ± SD, n = 4 biologically independent samples. Unpaired two-tailed t test was then performed and plotted: t = 0.3158, df = 6, P = 0.7629. d, Western blot of Gpx4-knockout Pfa1 cells overexpressing exogenous human WT or R152H GPX4, Gpx4-knockout Pfa1 cells overexpressing exonous murine WT or R152H mScarlet-tagged GPX4, and Gpx4-knockout HT1080 cells overexpressing exonous murine WT or R152H mScarlet-tagged GPX4 using GPX4 and GAPDH antibodies. Expression levels of GPX4 were quantified. Data are plotted as means ± SD, n = 3 biologically independent samples. Ordinary two-way ANOVA followed by Sidak’s multiple comparisons test was performed and P values were plotted: n = 3, DF = 12. Full scan image is shown in the Supplementary Information.
Extended Data Fig. 4 | Characterization of GPX4^{R152H} in cells and in vitro. a, HT1080 overexpressing exogenous WT or R152H GFP-GPX4 and a control line were tested for RSL3, ML162, and IKE sensitivity. Data are plotted as means ± SD, n = 3 biologically independent samples. b–c, SDS-PAGE gel of His-tagged GPX4^{U46C} and GPX4^{U46C,R152H} as stained by Coomasie Blue. Biologically independent duplicate experiments were performed and imaged. d–e, Distances between the catalytic triad in R152H variant were measured and labeled as compared to GPX4^{U46C} (PDB:2OBI). f, Shift of Lys48 away from the active site in the GPX4^{R152H} was plotted.
Extended Data Fig. 5 | Characterization of Lysine 48 mutants of GPX4 in cells and in vitro. a, Total GPX4 activity of HT1080 cells overexpressing GFP-GPX4WT, GFP-GPX4K48A, or GFP-GPX4K48L and a control line. Data are plotted as means ± SD of eleven biologically independent replicate experiments. Ordinary one-way ANOVA followed by Tukey’s multiple comparisons test was performed and P values were plotted, n = 11, DF = 40. b, HT1080 overexpressing exogenous WT, K48A, or K48L GFP-GPX4 and a control line were tested for RSL3 sensitivity. Data are plotted as means ± SD of three biologically independent replicate experiments. c–d, SDS–PAGE gel of His-tagged GPX4U46C_K48A and GPX4U46C_K48L as stained by Coomasie Blue. Biologically independent duplicate (GPX4U46C_K48A) and quadruplicate (GPX4U46C_K48L) experiments were performed and imaged. e, The distances between catalytic residues Sec46 and Trp136 were recorded every 4.8 ps throughout MD simulations of GPX4WT (PDB: 6HN3), GPX4K48A, GPX4K48L, GPX4K48E, GPX4K48Q, and GPX4K48R. Representative data from three times 100 ns trajectories were plotted as means ± SD. Ordinary one-way ANOVA followed by Tukey’s multiple comparisons test was performed: n = 20835, DF = 125004, all P**** < 1x10^-20. f, Scheme illustrating the catalytic cycle of sulfur-containing variant of GPX4.
Extended Data Fig. 6 | Characterization of Lysine 48 mutants of GPX4 in silico. a, In silico docking of GSH to GPX4U46C, GPX4K48A, or GPX4K48L. Top covalent-docking pose of GSH on GPX4U46C (top left). 2D Ligand interaction diagram of GSH with GPX4U46C, GPX4K48A, or GPX4K48L in their individually top covalent-docking pose (top right and bottom panels). b, HT1080 overexpressing exogenous WT, K48A, or K48L GFP-GPX4 and a control line were tested for IKE sensitivity. Data are plotted as means ± SD of three biologically independent replicate experiments.
Extended Data Fig. 7 | Investigation of GPX4 degradation mechanism after treatment with ferroptosis inducers. a, HT1080 overexpressing exogenous WT or R152H GFP-GPX4 and a control line were tested for FIN56 sensitivity. Data are plotted as means ± SD, n = 3 biologically independent samples. b, Western blot of HT1080 OE GFP-GPX4WT and HT1080 OE GFP-GPX4R152H after treatment with ferroptosis inducers with GPX4 and GAPDH antibodies, with lanes arranged for cell line comparison. Triplicate experiments were repeated independently with similar results, which were shown in Extended Data Fig. 7c,e,f. c, Western blot of HT1080 OE GFP-GPX4WT and HT1080 OE GFP-GPX4R152H after treatment with ferroptosis inducers with GPX4 and GAPDH antibodies, with lanes arranged for ferroptosis inducer comparison. Triplicate experiments were repeated independently with similar results, which were shown in Extended Data Fig. 7b,e,f. d, The endogenous GPX4 in HT1080 OE GFP-R152H-GPX4 were tested for vulnerability to the degradation induced by RSL3, ML162, FIN56, and IKE. Data are plotted as means with range of two biologically independent experiments. The corresponding blots are shown in Extended Data Fig. 7b,c. e, Western blot of HT1080 OE GFP-GPX4WT after treatment with ferroptosis inducers with GPX4 and GAPDH antibodies. Triplicate experiments were repeated independently with similar results, which were shown in Extended Data Fig. 7b,c. f, Western blot of HT1080 OE GFP-GPX4R152H after treatment with ferroptosis inducers using GPX4 and GAPDH antibodies. Triplicate experiments were repeated independently with similar results, which were shown in Extended Data Fig. 7b,c.
Extended Data Fig. 8 | Kinetic and mutagenesis study of GPX4 degradation mechanism after treatment with ferroptosis inducers. a, HT1080 OE GFP-GPX4WT and HT1080 OE GFP-GPX4R152H were treated with 4 µM RSL3, 30 µg/ml cycloheximide, and 100 µM a-Tocopherol for 0, 2, 4, or 6 hours before Western Blot analysis of GPX4 and GADPH. Biologically independent duplicate experiments were performed and imaged. b, Western blot of HT1080 OE GPX4K125R_K127R after treatments with ferroptosis inducers using GPX4 and GAPDH antibodies. Duplicate experiments were performed and imaged. c, The endogenous GPX4 in HT1080 OE GFP-K125R-K127R-GPX4 were tested for vulnerability to the degradation induced by RSL3, ML162, FIN56, and IKE. Data are plotted as means with range of two biologically independent experiments. The corresponding blots are shown in Extended Data Fig. 8b.
Extended Data Fig. 9 | Proof-of-concept treatments were tested on patient fibroblasts and Pfa1 cells, which were knocked out of endogenous GPX4 and transfected to overexpress human mScarlet-tagged GPX4WT (red) or GPX4R152H (blue). a–b, Supplementation of methyl-seleno-cysteine and N-acetyl-cysteine were tested as proof-of-concept treatments on the patient and control fibroblasts. Data are plotted as means ± SD (n = 3 biologically independent samples). c, Viability was normalized to the corresponding DMSO control. Data are plotted as means ± SD (n = 3 biologically independent samples). See Supplementary Note for effects of α-tocopherol.
Extended Data Fig. 10 | Proof-of-concept treatments were validated in Pfa1 cells, which were knocked out of endogenous GPX4 and transfected to overexpress murine mScarlet-tagged GPX4WT (red) or GPX4R152H (blue). Viability was normalized to the corresponding DMSO control. Data are plotted as means ± SD (n = 3 biologically independent samples). See Supplementary Note for effects of α-tocopherol.
Reporting Summary

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☐ A description of all covariates tested

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☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

Give P values as exact values whenever suitable.

☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection: Schrodinger Maestro (v.12.4.079) was used for computational modeling; The geometry of each crystal structure was fixed using programs XtalView (v4-1) and COOT (v0.8.9.2), and refined by Phenix (v1.17.1-3660).

Data analysis: ImageJ (v1.51) was used for Western Blot quantification; GraphPad Prism 9 was used for AUC analysis, line fitting, statistical analysis, and data plotting; The quantification of the immuno-fluorescence intensity was done using CellProfiler (v.3.1.8). Protein Thermal ShiftTM (v1.3) was used to analyze protein melting temperature.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Structural data for GPY4 has been deposited in the Protein Data Bank (PDB), with accession codes PDB IDs: 7L8K, 7L8L, 7L8M, 7L8R, and 7L8Q. These structures will be made publicly available upon publication. Publicly available datasets used in this study are: PDB IDs: 20B1, 6HN3. Source data are provided with this paper.
Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

Sample size calculations were not performed. Sample sizes for quantitative experiments are at least n=3. The only exception is western blot quantifications for protein degradation study, which were performed in duplicates. In all cases, these sample sizes were selected because the effect sizes were expected to be large enough to obtain statistical significance from small n values based on preliminary pilot experiments and our prior experiences with similar experiments.

Data exclusions

No data were excluded for analysis.

Replication

To ensure reproducibility of experimental findings, each assay was performed at least two times to confirm the results. In particular, at least three times molecular dynamic (MD) simulations with random seeding were performed for each system (Fig. 1c, d, 3e, g, Extended Data Fig. 2, 5e). Cell viability experiments were performed with at least three distinct replicates for each treatment group (Fig. 2d, 3b, h, 4a, 5c, 6, Extended Data Fig. 4a, 5b, 6c, 7a, 9, 10). GPX4 biochemical assay were carried out with at least three biological replicates for each treatment and these data were used to calculate mean values (Fig. 2a, c, 3a, 5b, Extended Data Fig. 3b, 5a). Western blot quantifications for protein level determinations (stand-alone study) were at least based on biological triplicates for each condition (Fig. 5a, Extended Data Fig. 3c, d). Western blot quantifications for degradation study (comparative study) were at least based on biological duplicates for each treatment (Fig. 4b-f, Extended Data Fig. 7b-f, 8). All above and additional replication information was stated in the legends of corresponding figures.

Randomization

Replicate molecular dynamic (MD) simulations were performed technically with random seedings in the computation program setting to generate diverse states of the protein structures being simulated. Imaging area of fibroblast cells under confocal microscope is randomly selected for unbiased analysis. Randomization was not relevant to other experiments, quantifications of which at an ensemble level are not subject to biased interpretation regardless of randomization in sample allocation.

Blinding

Blinding was not perform because the data we analyzed are not subject to biased interpretation.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☒  | Antibodies            |
| ☒  | Eukaryotic cell lines |
| ☒  | Palaeontology and archaeology |
| ☒  | Animals and other organisms |
| ☒  | Human research participants |
| ☒  | Clinical data         |
| ☒  | Dual use research of concern |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒  | ChiP-seq              |
| ☒  | Flow cytometry        |
| ☒  | MRI-based neuroimaging |

Antibodies

Antibodies used

All antibodies were obtained from commercial vendors. GPX4 [for WB, Abcam, ab125066], GPX4 [for IF, Santa Cruz, sc-166570], GFP (Santa Cruz, sc-9996), actin (Cell Signaling, D18C11), GAPDH [Santa Cruz, sc-47724], goat anti-mouse IgG (H+L) Secondary Antibody conjugated with Alexa Fluor 594 [for IF, Thermo Fisher Scientific Cat# A-11032, RRID:AB_2534091], IGDye® 800CW Goat anti-Rabbit IgG Secondary Antibody [for WB, LI-COR, #926-33221] and Goat anti Mouse IgG (H+L) Secondary Antibody conjugated with Alexa Fluor® 680 [for WB, Thermo Fisher Scientific Cat# A-21057.7]

Validation

1. GPX4 [for WB, Abcam, ab125066] was knockdown validated for WB application for mouse and human (referenced by 165 publications, including Chen C et al. Cell Death Dis. 12:65, 2021).
2. GPX4 [for IF, Santa Cruz, sc-166570] was validated for detection of GPX-4 of mouse, rat and human origin by WB, IF, IHC(P) and ELSA (referred by 24 publications, including Yamada, N., et al. 2020. Cell Death Dis. 11: 144.)
3. GFP (Santa Cruz, sc-9996) was validated for detection of GFP and GFP mutant fusion proteins (not species-specific by its nature) by
### Eukaryotic cell lines

**Cell line source(s)**

1. HT-1080 and Pfa-1 cells were obtained from ATCC. 2. Human fibroblast cell line RAG01 and RAG02 were developed from patient with homozygous R152H variant and his parent with heterozygous R152H variant. RAG01 and RAG02 cell lines are available for both commercial and academic use through CureGPX4.org, a patient organization dedicated to finding a treatment for SSMD disease.

**Authentication**

HT1080 and Pfa-1 cells were cells were from ATCC with authentication. The authentication was performed by morphology check under microscopes and growth curve analysis. Human fibroblast cells were developed from research participants by RUCDR Infinite Biologics, then directly allocated to the authors, and tested without further authentication.

**Mycoplasma contamination**

We confirm that all cells were tested as mycoplasma negative.

**Commonly misidentified lines**

(See [L1ACC register](https://example.com))

No commonly misidentified cell lines were used in the study.

### Human research participants

**Population characteristics**

Whole-exome sequencing (WES) was performed on three patients with SSMD features, in which homozygous R152H variant of GPX4 was recurrently identified. Ages and gender of the three patients were summarized below:

- 9 months old for Patient 1 (male, May 2019), 7 years old for Patient 2 (male, Dec 2015), and 10 months old for Patient 3 (female, Dec 2015).
- (The above ages are as of sequencing, with sequencing time denoted)

- Human fibroblast cell line RAG01 and RAG02 were developed from Patient 1 with homozygous R152H variant and his parent with heterozygous R152H variant:
  - RAG01 - 22 months old, male, Patient 1
  - RAG02 - 31 years old, male, parent of Patient 1, control
- (The above ages are as of sample collection)

**Recruitment**

- Patient 1 (Family 1) was transferred to Seattle Children’s Hospital (Seattle, WA, USA) at day of life 6, when he was evaluated and found to have multiple congenital abnormalities. Later as SSMD features were observed on Patient 1, WES sequencing of Patient 1 was performed and a homozygous R152H variant in GPX4 was then identified. He was therefore recruited to this study.

- Patient 2 and 3 (Family 2) were presented to the Biochemical Genetics Clinic (San Diego, CA, USA) for additional diagnostic evaluation after WES was obtained. Considering their SSMD clinical features and the concurrent R152H variant in GPX4, they were therefore recruited to this study.

- As of the time of this study, Family 1 and 2 were the only families with patients of homozygous R152H variant known to the authors. They were therefore all included into this study. No self-selection/exclusion of human research participant or related bias was presented.

**Ethics oversight**

All three patients and their families (Patient 1 from Family 1, and Patient 2 and 3 from Family 2) gave informed consent for genetic and clinical investigation. Both families were enrolled in an institutional approved study of children with undiagnosed neurogenetic disorders (Genomic Sequencing in Neurological Disorders research protocol, UCSF IRB #170437). Tissue samplings (including skin biopsy for the development of fibroblasts RAG01 and RAG02) and clinical observations of Patient 1 and his parent were approved by IRB #000002259 (Molecular and Biochemical Analysis of Metabolic Disorders, University of Washington). Study of GPX4 variant in the human fibroblast samples was approved by IRB #AAA59249 (Evaluation of GPX4 variant activity in fibroblasts, Columbia University). Compensations were not provided.

Note that full information on the approval of the study protocol must also be provided in the manuscript.