Phenotypic assays in yeast and zebrafish reveal drugs that rescue ATP13A2 deficiency

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Mutations in ATP13A2 (PARK9) are causally linked to the rare neurodegenerative disorders Kufor-Rakeb syndrome, hereditary spastic paraplegia and neuronal ceroid lipofuscinosis. This suggests that ATP13A2, a lysosomal cation-transporting ATPase, plays a crucial role in neuronal cells. The heterogeneity of the clinical spectrum of ATP13A2-associated disorders is not yet well understood and currently, these diseases remain without effective treatment. Interestingly, ATP13A2 is widely conserved among eukaryotes, and the yeast model for ATP13A2 deficiency was the first to indicate a role in heavy metal homeostasis, which was later confirmed in human cells. In this study, we show that the deletion of YPK9 (the yeast orthologue of ATP13A2) in Saccharomyces cerevisiae leads to growth impairment in the presence of Zn\(^{2+}\), Mn\(^{2+}\), Co\(^{2+}\) and Ni\(^{2+}\), with the strongest phenotype being observed in the presence of zinc. Using the ypk9Δ mutant, we developed a high-throughput growth rescue screen based on the Zn\(^{2+}\) sensitivity phenotype. Screening of two libraries of Food and Drug Administration-approved drugs identified 11 compounds that rescued growth. Subsequently, we generated a zebrafish model for ATP13A2 deficiency and found that both partial and complete loss of atp13a2 function led to increased sensitivity to Mn\(^{2+}\). Based on this phenotype, we confirmed two of the drugs found in the yeast screen to also exert a rescue effect in zebrafish—N-acetylcysteine, a potent antioxidant, and furaltadone, a nitrofuran antibiotic. This study further supports that combining the high-throughput screening capacity of yeast with rapid in vivo drug testing in zebrafish can represent an efficient drug repurposing strategy in the context of rare inherited disorders involving conserved genes. This work also deepens the understanding of the role of ATP13A2 in heavy metal detoxification and provides a new in vivo model for investigating ATP13A2 deficiency.
Introduction
ATP13A2, also known as PARK9 or CLN12 (Ramirez et al., 2006; Bras et al., 2012), belongs to the poorly characterized P5-type ATPase family of transport proteins (van Veen et al., 2014). The ATP13A2 gene encodes a lysosomal and late endosomal transmembrane protein of 1180 amino acids involved in cation transport, whose precise molecular function remains unknown. ATP13A2 is widely expressed by many neuronal populations, and more particularly in the dopaminergic neurons of the substantia nigra (Schultheis et al., 2004; Ramirez et al., 2006). Recessive mutations in the ATP13A2 gene were initially described to cause Kufor-Rakeb syndrome (KRS; OMIM# 606693), an atypical juvenile Parkinsonism with dementia and progressive brain atrophy (Ramirez et al., 2006). However, with the advent of new sequencing technologies and bioinformatics tools, the genetic and clinical spectra of ATP13A2 deficiencies have been expanded. Namely, ATP13A2 mutations have been additionally causally associated with a juvenile form of neuronal ceroid lipofuscinosis (OMIM# 606693; Farias et al., 2011; Bras et al., 2012) and a complicated form of hereditary spastic paraplegia-78 (OMIM# 617225; Kara et al., 2016; Estrada-Cuzcano et al., 2017). Juvenile neuronal ceroid lipofuscinosis is classified as a fatal lysosomal storage disorder characterized by the accumulation of an autofluorescent hydrophobic material called lipofuscin (Palmer et al., 1992). In contrast, hereditary spastic paraplegia-78 is considered as an adult neurodegenerative disorder, in which a main clinical feature is the progressive spasticity of the lower limbs due to axonopathy of the corticospinal neurons (Estrada-Cuzcano et al., 2017). Even more recently, the phenotypic spectrum associated with variants in the ATP13A2 gene was further expanded to also include a juvenile-onset form of amyotrophic lateral sclerosis (Spataro et al., 2019).

Although these four neurodegenerative diseases differ substantially from a clinical point of view, the cellular phenotypes associated with ATP13A2 deficiency converge. Studies in fibroblasts derived from KRS and hereditary spastic paraplegia-78 patients showed similar lysosomal and mitochondrial dysfunction phenotypes (Grunewald et al., 2012; Usenovic et al., 2012b; Estrada-Cuzcano et al., 2017). Furthermore, animal models of juvenile neuronal ceroid lipofuscinosis and KRS both...
featured age-related motor dysfunction, protein aggregation and lipofuscinosis (Farias et al., 2011; Wohlke et al., 2011; Schultheis et al., 2013; Kett et al., 2015). Interestingly, the ATP13A2 p.(T512I) missense variant has been described in patients diagnosed with KRS and hereditary spastic paraplegia-78, suggesting that environmental factors can determine the development of the clinical phenotype (Dehay et al., 2012; Usenovic et al., 2012a; Estrada-Cuzcano et al., 2017).

In fact, ATP13A2 polymorphisms have been identified in one study as potential risk markers for neurotoxic effects of manganese in humans and two KRS patients have been reported so far with increased iron levels in the brain (Behrens et al., 2010; Schneider et al., 2010; Rentschler et al., 2012). These two metals are currently the major metals associated with secondary Parkinsonism and increasing evidence suggests that heavy metal exposure is involved in several neurodegenerative conditions (Grubman et al., 2014; Cicero et al., 2017). Interestingly, the deletion of YPK9 (yeast orthologue of ATP13A2) in Saccharomyces cerevisiae led to decreased resistance against heavy metal toxicity (Gitler et al., 2009; Schmidt et al., 2009). The link between ATP13A2 and heavy metal homeostasis has received further support by subsequent studies in various mammalian cell models (Tan et al., 2011; Kong et al., 2014; Park et al., 2014).

Despite the strong evidence for a role of ATP13A2 in heavy metal homeostasis, the precise function of ATP13A2 remains unclear and there is currently no effective treatment to cure or reverse the symptoms of any of the ATP13A2-associated disorders. Others have previously shown that a 2-step phenotypic drug-screening approach in a more simple followed by a more complex model organism can be a promising strategy to identify potential lead compounds for inherited human disorders (Cotticelli et al., 2012; Soma et al., 2018; Vincent et al., 2018). Similarly, we took advantage of the apparent functional conservation of ATP13A2 across species to develop a drug-screening pipeline combining yeast (S. cerevisiae) and zebrafish (Danio rerio) models of ATP13A2 deficiency. Rapid growth, low cultivation costs and good reproducibility make budding yeast an excellent eukaryotic cell model for performing high-throughput (HTP) primary drug screens. HTP phenotypic screens in yeast models have, for instance, revealed compounds protecting against types of proteotoxicity relevant for human neurodegenerative disorders (Tardiff et al., 2012, 2013; Vincent et al., 2018). In this study, we screened >2500 compounds, including many approved drugs, for their ability to rescue growth in yeast yp9Δ strains in the presence of toxic levels of zinc. Eleven validated hits were further tested for their ability to rescue a decreased manganese resistance phenotype identified in atp13a2 deficient zebrafish larvae. As an outcome, N-acetylcysteine (NAC) and furaltadone (FTD), a nitrofuran antibiotic, emerged as compounds that actively protect against increased metal sensitivity induced by ATP13A2 deficiency, in both yeast and zebrafish models. More generally, our study further supports that coupling large-scale primary drug screening in yeast followed by a more focused validation screen in the whole-organism zebrafish model represents a powerful approach to discover lead compounds for inherited human disorders involving conserved disease genes.

Materials and methods

Yeast strains

ypk9Δ strains were generated in the BY4742 and FY4 backgrounds. The BY4742ypk9Δ strain was obtained by crossing the BY4741ypk9Δ strain from the gene deletion collection (EUROSCARF) with wild-type (WT) BY4742, followed by tetrathid dissection to isolate the genotype of interest. The FY4ypk9Δ strain was created via PCR-mediated gene replacement by the kanMX4 cassette (Brachmann et al., 1998) in the FY4 parental strain, which was a kind gift from Joseph Schacherer. To generate rescue strains, the YPK9 gene was amplified from yeast genomic DNA and cloned into the pAG303GPD-ccdB plasmid using Gateway technology (Alberti et al., 2007). Both the recombinant and empty plasmids were integrated into the HIS3 locus of BY4742ypk9Δ cells and positive clones were selected on synthetic complete medium lacking histidine. To generate the yp9Δpdr5Δ double mutant, the PDR5 gene was replaced by the URA3 marker in the BY4742ypk9Δ strain, followed by selection on synthetic complete medium lacking uracil. Transformations were performed using a standard polyethylene glycol/lithium acetate protocol as previously described (Gietz and Woods, 2002), and correct gene disruption or gene integration was verified by PCR. The strains, plasmids and primers used in this study are listed in Supplementary Tables 1–3.

Yeast cultivation media and growth conditions

Rich media (YPD) used for yeast transformation experiments contained 10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose. For all other cultivations in this study (metal ion measurements, growth phenotyping and drug screening), yeast cells were grown in YNB (6.7 g/l yeast nitrogen base without amino acids, 5 g/l ammonium sulfate, pH 5) supplemented with 20 g/l glucose, 0.08 g/l histidine, uracil, methionine, lysine and 0.24 g/l leucine. When needed for selection, the media were supplemented with geneticin (200 μg/ml). Metal solutions were sterilized by filtration and added at indicated concentrations to YNB medium.

Growth phenotyping assay in yeast

Yeast growth phenotyping was performed as previously described (Jung et al., 2015). Growth rate, final biomass
and lag time were analysed using the GATHODE software (Jung et al., 2015). The growth conditions tested in this study are listed in Supplementary Table 4.

Drug screening in yeast

Two fresh single colonies of the ypklΔpdr5Δ strain were inoculated from solid YPD plates into 5 ml YNB media with specific supplementation (as described above) and incubated overnight with shaking (200 rpm) at 30°C. After 16 h, 400 μl of overnight culture was inoculated into 4 ml fresh media and cultivated until the optical density at 600 nm (OD600) reached about 0.5. Compound plates were prepared by dispensing 25 μl growth media containing 15 mM ZnCl₂ into sterile 384-well microplates using an electronic 384-channel VIAFLO pipette (TECAN). The first and last two columns and primer sequences are given in the Supplementary material.

Metal ion measurements by inductively coupled plasma—mass spectrometry

Intracellular Zn²⁺ concentrations were measured by inductively coupled plasma—mass spectrometry (Agilent 7900) in samples prepared from three independent yeast cultivations as described in the Supplementary material.

Zinquin chelation assay

A solution of 20 μM Zinquin (Sigma-Aldrich) was mixed with 5, 10, 25 and 50 μM of selected drugs with or without 5 μM ZnCl₂ in phosphate-buffered saline at pH 7.0. Mixtures were incubated for 1 h at 25°C and shaking at 300 rpm. The samples were then transferred into a 96-well plate for fluorescence measurements in a microplate reader, at an excitation wavelength of 370 nm and an emission wavelength of 490 nm. Three independent replicates were measured per condition tested. The fluorescence intensities of the ZnCl₂-containing samples were background-corrected by subtracting the fluorescence intensity of the corresponding samples incubated in the absence of ZnCl₂.

Zebrafish strains

The atp13a2sa18624+/- and atp13a2sa14250+/- zebrafish lines (TLF background, F3 generation) were obtained from the European Zebrafish Resource Center and kept in our aquatic facility according to standard protocols (Westerfield, 2000). These atp13a2 lines were further out-crossed with WT (AB) or nacre lines.

Zebrafish embryos were obtained by natural spawning and then incubated in the dark at 28°C, in Danieau’s solution [17 mM NaCl, 2 mM KCl, 0.12 mM MgSO₄, 1.8 mM Ca(NO₃)₂, 1.5 mM HEPES pH 7.5 and 1.2 μM methylene blue]. If needed, to inhibit pigmentation, 24 h post-fertilization embryos were incubated in Danieau’s solution with 0.2 mM 1-phenyl-2-thiourea (PTU, Sigma). For live imaging, larvae were anaesthetized with buffered MS222 (0.016% w/v).

Ethics statement concerning zebrafish manipulations

The Aquatic Facility at the Luxembourg Centre for Systems Biomedicine is registered as an authorized breeder, supplier and user of zebrafish with Gran-Ducal decree of 20 January 2016. All practices involving zebrafish were performed in accordance with European laws, guidelines and policies for animal experimentation, housing and care (European Directive 2010/63/EU on the protection of animals used for scientific purposes). Authorization number LUPA 2017/04 allowed the performance of fin biopsies for genotyping purposes. Besides this, the present study did not involve any additional procedures within the meaning of Article 3 of Directive 2010/63/EU and as such it was not subjected to authorization by an ethics committee.

Genotyping of zebrafish lines

The atp13a2sa18624+/- and atp13a2sa14250+/- lines were fin-clipped and genotyped by PCR followed by AluI restriction analysis or sequencing, respectively. Detailed protocols and primer sequences are given in the Supplementary material and Supplementary Table 3.

Gene knockdown in zebrafish using morpholino antisense oligonucleotides

Translation and splice-blocking morpholinos (MOs) for atp13a2 and non-targeting control MOs were purchased from Gene Tools. Manual microinjections of MOs were performed as previously described (Rosen et al., 2009).
Morpholino sequences and the microinjection procedure are provided in the Supplementary material.

RNA extraction and quantitative PCR to study the tissular distribution of zebrafish atp13a2

Gene expression was analysed by quantitative PCR (qPCR) using the LightCycler480 (Roche). For each tissue, qPCR reactions were run for three biological replicates and two technical replicates were performed for each qPCR reaction. Expression levels of atp13a2 in indicated tissues were calculated relative to the eye tissue using the 2^−ΔΔCT method (Livak and Schmittgen, 2001). ef1α and rpl13a were used as reference genes for normalization. Tissular RNA extraction and cDNA synthesis protocols are detailed in the Supplementary material and the qPCR primer sequences are listed in Supplementary Table 3.

Heavy metal toxicity assay and drug screening in zebrafish

Two days post-fertilization (dpf) atp13a2−/− mutant embryos or atp13a2 knockdown morphants were incubated in a 48-well plate with Danieau’s solution containing varying concentrations of MnCl₂. The Mn²⁺-containing solution was renewed after 24 h and the toxicity assessed at 5 dpf. Experiments were performed with 12 larvae per well in quadruplet (n = 48) for each tested condition. For the drug screening, a similar procedure was applied but with drugs or vehicle (DMSO or water) added at 3 dpf and here experiments were performed with 12 or 15 larvae per well in triplicate. For consistency in the phenotypic scoring, all the experiments were performed by a single person, but the final hit confirmation for NAC and FTD in zebrafish was performed in ‘single-blinded’ experiments, in which the experimentalist did not know which larvae received which treatment, to avoid biased results.

Statistical analysis

All data are presented as means ± standard deviations (SDs). The statistical tests used for data analysis are described in the corresponding figure legends. Two-way ANOVA analyses with the respective post hoc tests were performed using the GraphPad Prism software (version 8.2.0) and extended statistics for the ANOVA tests are provided in Supplementary Table 5. The likelihood ratios for the zebrafish experiments were calculated using RStudio (version 1.0.143).

Data availability

The authors confirm that the data supporting the findings of this study are nearly fully available within the article and its supplementary documents. Additional supporting data are available from the corresponding author upon request.

Results

Deletion of the ATP13A2 homologue in yeast triggers metal hypersensitivity

Under standard yeast cultivation conditions, ypk9Δ strains grew similarly to WT control strains. In order to identify a phenotype suitable for implementing an HTP drug screen under YPK9 deficiency, we assayed the growth of WT and ypk9Δ strains in >200 different conditions in liquid microcultivation (Supplementary Table 4). For a subset of the growth conditions, including different carbon sources, pH values, stressors, drugs and defined concentrations of various heavy metals, we tested WT and knockout strains in two different genetic backgrounds (BY4742 and FY4) and additionally a YPK9 rescue strain (YPK9OE) in the BY4742 background (Fig. 1A and Supplementary Table 4). In both backgrounds, YPK9 deficiency led to a slowed growth phenotype only in the presence of certain metals (Zn²⁺, Mn²⁺, Ni²⁺ and Co²⁺, but not Ca²⁺, Li⁺ and Cu²⁺) in the cultivation medium. Although we were not able to detect a previously reported growth defect of the ypk9Δ strain in the presence of Cd²⁺ (Schmidt et al., 2009), we found that over-expression of YPK9 enhanced growth under Cd²⁺ exposure (Fig. 1A). Subsequently, high-density growth curves (allowing for specific growth rate and yield of biomass calculation) were determined in the presence of the divalent metals Zn²⁺, Mn²⁺, Ni²⁺ and Co²⁺ for the WT, ypk9Δ and YPK9OE strains (BY4742 background), at concentrations that we had previously shown to significantly impair the growth of WT and YPK9OE strains in different carbon sources, pH values, stressors, drugs and Co²⁺ pools. In the absence of supplemented zinc, the WT, ypk9Δ and YPK9OE strains...
Figure 1 Large-scale phenotyping of ypk9Δ strains and validation of growth conditions for drug screening. (A) Heat map representing OD₆₀₀ values measured 48 h after the inoculation of liquid microcultures in the indicated conditions with WT, ypk9Δ and YPK9 overexpression (YPK9OE) strains (BY4742 and/or FY4 background). The deletion of YPK9 resulted in growth defects in media containing Co²⁺, Mn²⁺, Zn²⁺ and Ni²⁺ (red arrows) and YPK9 overexpression rescued the growth defects (red and blue arrows). (B) Representative growth curves of WT, ypk9Δ and YPK9OE strains (BY4742 background) obtained in an independent experiment in the presence of Zn²⁺, Mn²⁺, Ni²⁺ and Co²⁺ at the indicated concentrations. (C) Biomass (at 48 h) and growth rates determined from the growth curves shown in B. (D) Growth curves of the same strains in minimal growth medium supplemented or not with 5 mM ZnCl₂. Red boxes indicate the sampling points for metal
showed similar intracellular Zn\(^{2+}\) concentrations (≈80–90 μg/g dry weight) at the two measurement time points (early post-diauxic and stationary phase; Fig. 1D and E). Supplementation of 5 mM ZnCl\(_2\) increased the intracellular Zn\(^{2+}\) concentrations about 100 times (≈7000–8000 μg/g dry weight). Interestingly, WT cells, but not yp9kΔ cells, were able to reduce the intracellular Zn\(^{2+}\) levels by about 2-fold; this decrease was even more drastic (14-fold) when YPK9 was overexpressed (Fig. 1E). These results suggest that YPK9 is not essential for cell proliferation in the presence of subtoxic levels of Zn\(^{2+}\), but that it plays an important role in the stress response induced by overexposure to this and probably some other metals in post-mitotic and/or aged cells. The observation that YPK9 overexpression led to intracellular Zn\(^{2+}\) levels that are lower than in the WT strain is consistent with the growth assays under divalent metal overexposure, where YPK9 overexpression enhanced growth rate and yield of biomass beyond the WT parameters (Fig. 1B and C), indicating that basal YPK9 expression levels do not provide optimal protection against metal ion stress.

### High-throughput compound screen in an ATP13A2-deficient yeast model

HTP drug screening in the yeast model was performed in the 384-well microplate format where cells were cultivated in the presence of 15 mM ZnCl\(_2\) at 30°C, with OD\(_{600}\) measurements being taken just after inoculation as well as 48 h and 96 h later. To increase the probability of positive hits in the presence of lower drug concentrations, we additionally deleted the PDR5 gene in our yp9kΔ strain. PDR5 encodes the most pleiotropic drug export pump in budding yeast and its deletion has been shown to increase the susceptibility to externally added compounds (Rogers et al., 2001). We confirmed that PDR5 deletion did not cause any additional growth impairment in standard conditions or in the presence of ZnCl\(_2\), be it in the WT or in the yp9kΔ background (Supplementary Fig. 1B). For assay quality assessment, the Z’-factor was calculated for each tested plate based on negative and positive control wells containing DMSO and EDTA, respectively. EDTA restores WT growth by extracellular zinc chelation (Supplementary Fig. 1C) and, given the absence of any currently known drug to treat ATP13A2 deficiencies, served as a reference point for hit identification. In addition, cell vitality was tested in control wells containing medium devoid of Zn\(^{2+}\) and each plate also contained microbial contamination control wells (Fig. 2A).

To identify lead compounds with potential therapeutic value for ATP13A2 deficiencies, the Prestwick and Tocris chemical libraries, which contain 1280 and 1273 compounds, respectively (of which 815 and 160 correspond to FDA/EMA-approved drugs), were screened using the yeast growth rescue assay. In total, 117 compounds are contained in both libraries and were thus tested twice. In total, 2553 compounds (of which 2436 unique compounds) were tested in duplicate and at two different concentrations (10 and 100 μM). In total, 24 compounds were identified as positive hits in both replicates (Fig. 2B and Supplementary Fig. 2 and Table 6). Of these, 17 compounds tested positive at one concentration only and seven compounds were positive at both concentrations. Of the 24 hit compounds, 22 compounds were re-tested (only three of the five cephalosporin antibiotic hits were further tested so far) and 11 could be validated by showing a reproducible protective effect against Zn\(^{2+}\)-induced toxicity in a dose-response assay in the yp9kΔpdr5Δ strain, resulting in a validated hit rate of 0.5% (Fig. 2C). These 11 compounds can be grouped into three categories: (i) non-FDA-approved drugs (VU152100, VU10010, ML-365 and AMG-9810), (ii) antibiotics (moxalactam, cefmetazole, cefazolin and FTD) and (iii) direct or indirect chelators [NAC, deferoxamine and tetraethylenepentamine (TEPA)].

As seen in Fig. 2C, all validated drugs slightly alleviated the Zn\(^{2+}\)-induced stress in WT cells, at least at the lower concentrations tested. In contrast, a stronger and dose-dependent positive effect was observed in YPK9-deficient cells. With the non-FDA-/EMA-approved compounds, virtually full rescue effects were reached at relatively low concentrations. These drugs precipitated at relatively high concentrations (>300 μM) and were, therefore, not tested at higher doses. Moreover, foam formation in microcultivations containing AMG9810 interfered with the OD measurements, leading to high variations in the results obtained with this compound. The remaining (FDA-approved) drugs were more soluble in water and were tested at higher concentrations without toxic effect, except for two antibiotics (FTD and cefazolin) that impaired growth of both WT and YPK9 deficient cells at the higher doses tested. Within the antibiotic group, containing cephalosporin antibiotics from different generations, cefmetazole exerted the strongest rescue effect. In the chelator group, a nearly full rescue effect was reached with NAC and TEPA, whereas only a moderate effect was observed with the lower concentrations tested for deferoxamine.
Figure 2 Drug screening of the Prestwick and Tocris chemical libraries in a yeast ATP13A2 deficiency model. (A) Pipeline for drug screening in 384-well plate format. Positive controls (4.5 mM EDTA, n = 16) and negative controls (0.1 and 1% DMSO, n = 16) were alternated in the first and second columns of each plate. The last two columns of each plate were reserved for 16 vitality (no metal) and 16 contamination (no cell inoculation) control wells. Drugs (n = 2553) were tested at 10 and 100 μM in biological duplicates. OD₆₀₀ was measured 48 and 96 h after inoculation and standard statistical analysis for HTP drug-screening data was performed for each time point in order to determine the indicated parameters. (B) Z-scores for the screening plates with acceptable Z'-factor. From both libraries, 24 drugs were identified as positive hits (Z-score > cut-off value). Of those, 10 drugs were positive at 10 μM, 21 drugs at 100 μM and 14 were FDA-approved drugs. (C) From the 24 hits, 11 were validated in independent dose-response assays. Data shown are means ± SDs for three biological replicates. Statistically significant
Chelation activity of the yeast phenotypic hit compounds

The discrepancy between the concentrations at which the yeast phenotypic hit compounds at least started to exert rescue effects (10–100 μM) and the ZnCl₂ concentration present in the cultivation medium (15 mM) for the drug-screening and dose-response assays suggested that the main mechanism of action of the hit compounds was not extracellular metal ion chelation. To consolidate this assumption, a fluorometric chelation assay using zinquin was performed to assess the chelation activity of the hit compounds. Zinquin forms a fluorescent complex with Zn²⁺ ions and the complex is UV-excitable. As positive controls, we used EDTA and clioquinol, both well-known Zn²⁺ chelators. The strong chelator EDTA completely sequestered the Zn²⁺ (20 μM) bound to zinquin already at the lowest concentration tested (10 μM), whereas clioquinol showed an increasing chelation activity between 5 and 50 μM. Except for VU152100 and VU100010 (which interfered with the zinquin chelation assay, due to their strong inherent fluorescence), the chelation activity of all the validated yeast phenotypic hit compounds was assessed in this same concentration range (Fig. 3A). Only TEPA, an industrial iron chelator, showed a relatively strong Zn²⁺ chelation activity, comparable with clioquinol at the highest concentration tested. From the eight remaining compounds, only the nitrofuran antibiotic FTD operation were mated and their offspring (n = 37) were genotyped after 2 months; 35% of the progeny was found to be homozygous for the mutation, showing that atp13a2 deletion in zebrafish is not lethal during development (Fig. 4B). Furthermore, homozygous mutants were able to reach adulthood without any obvious morphological or behavioural abnormalities.

As ATP13A2 seems to play an important role in heavy metal homeostasis (this study; Tan et al., 2011; Park et al., 2014), we tested the effect of manganese on atp13a2(+/-) larvae. Exposure to MnCl₂ has previously been described to cause neurodegeneration in zebrafish, and in humans, it leads to manganism, a disorder featuring symptoms that resemble those of Parkinson’s diseases (Bakhavatsalam et al., 2014; Roth, 2014). In this study, we exposed zebrafish larvae to 2 and 3 mM MnCl₂ and compared the morphological abnormalities at 5 dpf. In the presence of Mn²⁺, WT and heterozygous larvae showed a moderate phenotype characterized mainly by an underdeveloped swimming bladder. In contrast, homozygous atp13a2 mutants were highly sensitive to Mn²⁺ and displayed multiple abnormalities, including pericardial oedemas, movement loss and spine curvature in addition to the underdevelopment of the swimming bladder.

animals in the gonads, the brain and the eyes (Supplementary Fig. 3), further supporting the potential relevance of zebrafish in ATP13A2-linked disease modelling. It should be noted that rpl13x is more stably expressed across tissues in both males and females than ef1x; therefore, we believe that the relative gene expression results based on the rpl13x reference gene are more reliable (McCurley and Callard, 2008; Xu et al., 2016).

Zebrafish lines carrying atp13a2 mutant alleles (atp13a2sa14250 or atp13a2sa18624) generated by N-ethyl-N-nitrosoourea mutagenesis (Howe et al., 2013) were obtained from the European Zebrafish Resource Center. Each allele carries a single non-sense mutation (2153T > A or 2457T > G) resulting in a premature stop codon in exon 20 or exon 22, respectively (Fig. 4A).

Both mutations can be predicted to cause a loss-of-function, as they lead to the elimination of important sequences of the P-domain, responsible for the binding of the Mg²⁺-ATP complex via Mg²⁺ coordination, as well as the transmembrane domains M5-M10 (Sorensen et al., 2010; Fig. 4B). The point mutation in the atp13a2sa18624 allele creates a new AluI restriction site, allowing to easily follow its transmission through generations by genotyping (Fig. 4B). We, therefore, focused on the atp13a2sa18624 line, which was backcrossed to a WT line to reduce unspecific mutations generated by N-ethyl-N-nitrosoourea mutagenesis. Heterozygotes of the F5 generation were mated and their offspring (n = 37) were genotyped after 2 months; 35% of the progeny was found to be homozygous for the mutation, showing that atp13a2 deletion in zebrafish is not lethal during development (Fig. 4B). Furthermore, homozygous mutants were able to reach adulthood without any obvious morphological or behavioural abnormalities.

atp13a2 loss-of-function leads to decreased resistance against manganese toxicity in zebrafish

As ATP13A2-related disorders will predictably respond mostly to drugs which are able to reach their target site within the central nervous system, we established an atp13a2 deficient zebrafish model to further validate our positive hit compounds in vivo. As the ATP13A2 protein, which shares 50% amino acid sequence identity between human and zebrafish, has been studied only very little in the latter model (Lopes da Fonseca et al., 2013; Spataro et al., 2019), we first analysed the tissue distribution of the atp13a2 transcript in adult fish using qPCR. atp13a2 showed the highest expression in both female and male
The homozygous mutant larvae also frequently featured darkening of the brain region, suggesting that this organ might be particularly affected by Mn\textsuperscript{2+} exposure in the absence of functional ATP13A2. Similar results were obtained in the second mutant line (atp13a2sa14250) upon exposure to Mn\textsuperscript{2+}. To uncover possible reasons for the darkening of the brain in mutant atp13a2sa18624/C0/C0 larvae, a TUNEL assay was performed in 5 dpf larvae exposed or not to Mn\textsuperscript{2+}. Strikingly, 64% of the homozygous atp13a2 mutants showed large apoptotic areas throughout the central nervous system (brain and dorsal spine) after exposure to Mn\textsuperscript{2+}. As for the yeast ypk9Δ model, these observations show that atp13a2 deficiency renders zebrafish larvae much more sensitive to manganese exposure, with the interesting additional information from the zebrafish model that this sensitivity seems to be particularly pronounced in nerve cells in vertebrates.

( Fig. 4C and Supplementary Fig. 4). The homozygous mutant larvae also frequently featured darkening of the brain region, suggesting that this organ might be particularly affected by Mn\textsuperscript{2+} exposure in the absence of functional ATP13A2. Similar results were obtained in the second mutant line (atp13a2sa14250) upon exposure to Mn\textsuperscript{2+} (Supplementary Fig. 5). To uncover possible reasons for the darkening of the brain in mutant atp13a2sa18624Δ/C0/C0 larvae, a TUNEL assay was performed in 5 dpf larvae exposed or not to Mn\textsuperscript{2+}. Strikingly, 64% of the homozygous atp13a2 mutants showed large apoptotic areas throughout the central nervous system (brain and dorsal spine) after exposure to Mn\textsuperscript{2+}, a phenotype that did not develop in the great majority of the WT control larvae in that same condition (Fig. 4D and E).

As for the yeast ypk9Δ model, these observations show that atp13a2 deficiency renders zebrafish larvae much more sensitive to manganese exposure, with the interesting additional information from the zebrafish model that this sensitivity seems to be particularly pronounced in nerve cells in vertebrates.

Given the strong zinc sensitivity phenotype observed in the yeast model, we also tested the effect of zinc exposure on zebrafish larvae. At lower concentrations of Zn\textsuperscript{2+} (up to 0.3 mM), we could not observe any dysmorphology or changes in locomotor behaviour in either control or homozygous atp13a2 mutant larvae (data not shown). However, at 1 mM ZnCl\textsubscript{2}, we observed higher lethality and strong tissue damage (often starting in the caudal region) in both the control and mutant larvae (Supplementary Fig. 6).

In a previous study, transient downregulation of atp13a2 by microinjection of an exon1-intron1 splice-blocking morpholino antisense oligonucleotide (e11-MO) was reported to be lethal in zebrafish larvae (Lopes da Fonseca et al., 2013). This suggested that compensatory mechanisms (not induced in the atp13a2 knockdown larvae) may explain the absence of an overt phenotype (under control conditions) of our atp13a2 knockout mutants. Another explanation for the apparently contradictory results could be a gene-independent toxicity of the e11-MO used previously and we started testing this MO by injection into our atp13a2sa18624Δ/C0/C0 and atp13a2sa14250Δ/C0/C0 mutant embryos. At 3 dpf, all embryos were dysmorphic and showed locomotor impairments. In contrast, homozygous mutants injected with a control morpholino did not display any abnormality, indeed suggesting a possible atp13a2 independent effect elicited by the e11-MO (Supplementary Fig. 7). Therefore, for validation of the Mn\textsuperscript{2+} phenotype in atp13a2 morphants (as
Figure 4 Genotyping and phenotyping of atp13a2−/− zebrafish mutants. (A) Schematic illustration of the zebrafish atp13a2 gene structure. Exons are represented as numbered boxes and homologous positions of human disease-associated mutations that are close to mutations present in the zebrafish mutant lines used in this study are indicated (in black, non-sense mutations in zebrafish mutant lines; in blue, KRS-associated mutations; in green, NCL-associated mutation). In red, important domains for the ATP13A2 function are also indicated. (B) atp13a2sa18624/+ heterozygous carriers were crossed and the offspring was raised to adulthood. AluI restriction analysis showed that 35% of the F6 population were homozygous mutants. Genomic DNA sequencing confirmed the expected mutations in heterozygous and homozygous zebrafish. (C) 2 dpf atp13a2sa18624/+ , atp13a2sa18624/− and atp13a2sa18624/−/− larvae were incubated without or with 2 and 3 mM MnCl2 for 3 days. All 5 dpf larvae incubated in the presence of MnCl2 lacked the swimming bladder (moderate phenotype), but homozygous atp13a2sa18624/−/− larvae, in addition, showed increased occurrence of pericardial oedemas, spine curvature, darkening of the brain tissue (strong phenotype).
opposed to mutants), we designed a translation (ATG-MO) and a different splice-blocking (e2i2-MO) morpholino to inhibit the expression and processing of newly synthesized atp13a2 mRNA, respectively (Supplementary Fig. 8A). No obvious phenotypes were observed up to 5 dpf upon injection of 8 ng of either morpholino into WT embryos (Supplementary Fig. 8B). The lack of antibodies against the zebrafish Atp13a2 protein prevented the verification of decreased Atp13a2 levels in the morphants generated by injection of the ATG-MO. However, the efficiency of our splice-blocking morpholino was confirmed at 3 dpf by qPCR and sequencing of cDNA from these morphants provided evidence for aberrant atp13a2 splicing leading to an early stop codon (Supplementary Fig. 8A).

Subsequently, we incubated atp13a2 morphant larvae (generated with ATG-MO and e2i2-MO) in the presence of 1 and 3 mM MnCl2 and analysed them morphologically at 5 dpf. atp13a2 morphants were substantially more sensitive to Mn2+ than the control larvae (Supplementary Fig. 8C) and surviving larvae displayed similar phenotypes to those previously described for the atp13a2 mutant lines (pericardial oedemas, darkening of areas in the brain, spine curvature; Supplementary Fig. 8D). Actually, atp13a2 morphants showed even higher sensitivity to Mn2+ compared with the mutant larvae as exposure to 1 mM Mn2+ had a similar impact on the morphants as exposure to 3 mM Mn2+ on the mutants. Morphants were generated from the commonly used AB zebrafish strain whereas the mutant lines had been created in the Tüpfel long fin strain. A different genetic background may, therefore, at least partially explain that a partial gene knockdown led to a stronger phenotype than a null mutation. Overall, the results obtained in the atp13a2 morphants (created with our newly designed MOs) and atp13a2 knockout models were, however, in good agreement and our observations suggest that functional ATP13A2 protects multicellular organisms against manganese toxicity, but that it is not essential for zebrafish development under standard conditions.

**Validation of yeast phenotypic hit compounds in atp13a2 knockout zebrafish**

To further validate the hits obtained in the primary yeast screen in a more complex organism, we proceeded to their *in vivo* testing in our atp13a2 zebrafish mutant line (except for the industrial chelator TEPA). For each compound class, the maximum tolerated concentration was first determined in WT embryos in the absence of Mn2+ and sublethal drug concentrations were then tested for their phenotypic rescue potential (Supplementary Fig. 9A). atp13a2sa18624+/− and atp13a2sa18624−/− larvae at 2 dpf were incubated in medium containing 3.5 mM MnCl2, followed 24 h later by an exchange for medium containing different concentrations of the drugs (0.1–1000 µM) in addition to MnCl2. Two of the 10 hit compounds tested (NAC and FTD) protected dose-dependently against Mn2+ toxicity, with an apparently more pronounced effect in mutant than in control larvae (5 dpf). Considering, for instance, the highest drug concentration tested (1 mM), >80% of the atp13a2a2 mutant larvae presented merely a moderate phenotype (absence of the swimming bladder) whereas >65% of these larvae either died or presented severe morphological abnormalities in the absence of drug (Fig. 5B and C). NAC and FTD also protected against Mn2+ toxicity in the WT larvae, but their relative effect was less pronounced in these animals given a weaker phenotypic impact of Mn2+ in the absence of the drug. The remaining drugs tested failed to rescue the phenotype induced by manganese exposure, be it in the WT or mutant larvae (Supplementary Fig. 9B).

**Discussion**

**ATP13A2 and heavy metal stress response in budding yeast and zebrafish**

In this study, of around 200 growth conditions tested for ATP13A2 deficient yeast models in two different genetic backgrounds, only the presence of Mn2+, Co2+, Zn2+, and Ni2+ led to significant growth impairments in both ypkl9Δ strains. We also found that, under Zn2+-repletion conditions, post-mitotic ypkl9Δ cells contained considerably higher concentrations of this ion than WT cells. Accordingly, human cells derived from the olfactory mucosa (hONs) of ATP13A2−/− patients showed decreased viability in the presence of Zn2+ (Kong et al., 2014; Park et al., 2014). Also, patient cells showed higher intracellular Zn2+ levels than control cells only when challenged with ZnCl2. Interestingly, vesicular zinc levels were, however, lower in the ATP13A2 deficient cells (Kong et al., 2014; Park et al., 2014). Together with our data, all studies indicate that ATP13A2 is critically involved in zinc compartmentalization or externalization under conditions where
storage compartments (vacuole/lysosomes) become over-loaded. Further supporting the involvement of ATP13A2 in metal detoxification, we observed that deletion or transient knockdown of \textit{atp13a2} in zebrafish caused increased sensitivity to MnCl\textsubscript{2} exposure, resulting in pericardial oedemas, spine curvature and higher mortality. As in the yeast model, we could only observe an obvious phenotype in zebrafish when we challenged the model with relatively high metal ion concentrations.

The main goal of this study was to perform a drug screen for ATP13A2 deficiencies, calling for acute exposure protocols yielding relevant phenotypes in a short time frame to allow for HTP screening. We did not evaluate long-term exposure to heavy metals, but considering that ATP13A2 deficiencies are linked to juvenile and adult rare neurodegenerative disorders, it might be that a slight dysregulation of heavy metal homeostasis leads to gradual accumulation and/or mis-localization of those metals over the years until the onset of disease symptoms.

Heavy metals have been found to be part of lipofuscins, a hallmark of NCLs, as well as of abnormal aggregates containing mainly α-synuclein in the brain of Parkinson’s disease patients (Terman and Brunk, 1998; Gardner et al., 2017). The fact that these features have also been observed in ATP13A2-deficient patients and animal models, combined with ample evidence for the role of ATP13A2 in heavy metal homeostasis, suggest that heavy metals may play a pivotal role in the pathology of ATP13A2-associated disorders. Notably, the clinical heterogeneity observed in ATP13A2 patients may result from varying interactions between environmental metal exposures and genetic factors (Rentschler et al., 2012).

**Primary and secondary drug screen for ATP13A2 deficiencies**

In the present study, we developed and combined two phenotypic drug-screening assays, using budding yeast...
and zebrafish to identify drugs that could be used to treat \( \text{ATP}13\text{A}2 \)-associated disorders. Currently, 32 pathogenic mutations have been described for \( \text{ATP}13\text{A}2 \) and most of these mutations appear to cause protein instability or functional disruption (Ramirez et al., 2006; Podhajska et al., 2012; Estrada-Cuzcano et al., 2017). We, therefore, opted for complete loss-of-function models, seeking to identify drugs with therapeutic potential for most of these disorders.

As the mechanism of action of \( \text{ATP}13\text{A}2 \) in heavy metal detoxification seems to be well-conserved (Gitler et al., 2009; Schmidt et al., 2009; Tan et al., 2011; Kong et al., 2014; Park et al., 2014), we used yeast as a HTP screening platform for chemicals that alleviate \( \text{Zn}^{2+} \)-induced stress in YPK9 deleted cells. We focused the screen on libraries containing in large part FDA- and EMA-approved drugs and tested in total 2553 chemical compounds. From the primary screening, 11 hits were validated in a concentration-response assay. For further validation of the hits, an \( \text{in vivo} \) disease model in zebrafish was established using \( \text{atp}13\text{a}2 \) mutant larvae exposed to \( \text{Mn}^{2+} \). In addition to this \( \text{in vivo} \) evaluation of compound efficacy, zebrafish larvae allow to concomitantly address drug safety and drug delivery through the blood-brain barrier, which is a major challenge in drug discovery for neurodegenerative disorders (Elicheiri et al., 2011; Kanungo et al., 2014; Zeng et al., 2017).

Of the 11 hits identified in the yeast-based primary screen, NAC and FTD were validated as being able to alleviate \( \text{Mn}^{2+} \) toxicity in the zebrafish-based secondary screen. NAC was initially prescribed for the treatment of paracetamol-induced hepatotoxicity by restoring hepatic concentrations of glutathione and also as a mucolytic agent (Bavarsad Shahrripour et al., 2014). However, in the last year, several studies have reported a neuroprotective function for NAC by increasing the glutathione levels (Bavarsad Shahrripour et al., 2014). Furthermore, glutathione deficiency has long been implicated in the development of Parkinson’s disease (Perry et al., 1982; Bjorklund et al., 2018). In fact, one ongoing clinical trial using NAC has been reported for Parkinson’s disease (Monti et al., 2015) and NAC treatment also seems to be beneficial for Alzheimer’s disease where it showed positive effects on some secondary outcome measures (cognitive tests; Adair et al., 2001). Interestingly, independently to this study, it has been reported that NAC mitigates \( \text{Zn}^{2+} \)- and \( \text{Fe}^{2+} \)-induced toxicity in \( \text{ATP}13\text{A}2 \) hONs and CHO cells (Park et al., 2014; Rinaldi et al., 2015). The positive effects of NAC in three different disease models for \( \text{ATP}13\text{A}2 \) deficiency, in addition to the described beneficial effects in Parkinson’s and Alzheimer’s disease, suggest that its nutritional supplementation could be of therapeutic value in \( \text{ATP}13\text{A}2 \)-associated disorders. Interestingly, another mucolytic agent, Ambroxol, has emerged recently as a drug with beneficial effects in Parkinson’s disease and Gaucher’s disease. Not only does Ambroxol seem to be a potent chaperone for glucocerebrosidase, the enzyme deficient in Gaucher patients, but it has been shown to promote exocytosis, as well as lysosomal and mitochondrial biogenesis (Magalhaes et al., 2018). It remains to be explored whether the beneficial effects of NAC in neurodegenerative disorders may be mediated at least in part through similar properties.

In contrast, little is known about FTD, a nitrofuran antibiotic primarily used for bacterial infections in poultry (Nouws et al., 1987). Currently, nitrofurans are prohibited in the EU for livestock production due to their potential carcinogenicity. However, nitrofuran antibiotics can still be used in humans for the treatment of infections of the urinary tract and the skin as well as bacterial diarrhoea (Guay, 2008; Vasheghani et al., 2008). Interestingly, although five members of this family of antibiotics were present in one of the libraries screened in this study, only FTD was identified as a hit. In comparison to other nitrofurans, FTD has an additional morpholinomethyl modification, which is retained in FTD-derived metabolites (AMOZ; Supplementary Fig. 10; Vass et al., 2008). In the zinquin chelation assay, FTD exhibited a slight chelation activity at high concentrations, suggesting that intracellular metal ion chelation by FTD and/or one or several of its biotransformation product(s) could be a mechanism of action. Although the use of FTD is currently controversial, our study suggests that this compound possesses some favourable features in the context of heavy metal exposure, more particularly in combination with \( \text{ATP}13\text{A}2 \) deficiency, and that it could be used for chemical lead optimization.

Chelating agents have been proposed as effective secondary antioxidants by stabilizing the oxidative form of heavy metals and thereby decreasing metal ion induced oxidative stress that may, for instance, cause cell death (Turan et al., 2016). Moreover, KRS belongs to the group of neurodegenerative disorders with brain iron accumulation and several \( \text{ATP}13\text{A}2 \) deficiency models, including the yeast and zebrafish models used here, strongly implicate this protein in the compartmentalization and externalization of heavy metals. Taken together, these findings suggest that chelation therapy could be beneficial for the treatment of \( \text{ATP}13\text{A}2 \)-associated disorders. Some clinical studies already support the therapeutic use of chelators for other neurodegenerative disorders, such as Friedreich’s ataxia (Boddaert et al., 2007; Dusek et al., 2016; Martin-Bastida et al., 2017). In this disease, the decrease of iron–sulphur clusters and haem formation cause iron accumulation in mitochondria, and administration of deferiprone to these patients over a 6-month period reduced iron content, resulting in improved clinical symptoms (Boddaert et al., 2007). However, larger clinical trials and studies are still needed, as chelation therapy remains controversial; important metals needed for normal cellular function could be unspecifically chelated, resulting in non-desired side effects (Nunez and Chanacuevas, 2018).
Most drugs identified in the yeast model failed to rescue the phenotype in the zebrafish model. Possible reasons for this could be: (i) a low penetration through the blood-brain barrier, (ii) a yeast-specific mechanism of action and/or (iii) a Zn\(^{2+}\)-specific effect. Indeed, cephalosporin antibiotics are known to have a generally poor blood-brain barrier penetration (Lutsar and Friedland, 2000). Our finding that all cephalosporin antibiotics (belonging to the class of \(\beta\)-lactam antibiotics) contained in the Prestwick library were identified as hits in the yeast-based primary screen is nevertheless noteworthy. It has been demonstrated that \(\beta\)-lactams confer neuroprotection by decreasing synaptic glutamate levels through the increase of glutamate uptake in the cells. This effect was shown to be beneficial in an SOD1-G93A transgenic mouse model of amyotrophic lateral sclerosis (Rothstein et al., 2005), a disease that has also been recently linked to ATP13A2-deficiency (Spataro et al., 2019). In the mouse model, ceftriaxone (a third-generation \(\beta\)-lactam not contained in the chemical libraries screened in this study) treatment delayed the loss of muscle strength and body weight. In addition, \(\beta\)-lactams are also known as metal chelators such as Zn\(^{2+}\), Cu\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\) (Mukherjee and Ghosh, 1995), although they did not show a high affinity for Zn\(^{2+}\) using our zinquin assay.

Other interesting hits were VU152100 and VU10010, two selective activators of the M4 muscarinic acetylcholine receptor, which were able to rescue the yeast phenotype in the micromolar range. M4 receptors are G protein-coupled receptors that play an important role in regulating midbrain dopaminergic activity; they are attractive entry points for the treatment of disorders involving altered dopaminergic function in the basal ganglia, including Parkinson’s disease (Brady et al., 2008). However, the yeast genome does not encode any protein with appreciable homology to the human M4 receptor. Similarly, two additional non-approved drugs identified as hits in the yeast screen, AMG9810 and ML363, bind to proteins (vanilloid TRPV1 receptor and TASK-1 channel, respectively; Gavva et al., 2005; Zou et al., 2010) that are not conserved in yeast. Understanding the mechanism by which these drugs alleviate the Zn\(^{2+}\) toxicity in the yeast ypk9Δ mutant thus requires further investigation.

The cellular protectant properties of the hits identified during our primary HTP screen demonstrate the strength of yeast for the identification of chemical suppressors of ATP13A2-associated disease phenotypes. However, in vivo secondary screening is clearly important for validating the bioactivity of the ‘primary’ hits in the context of a multicellular organism. In this study, we utilized the advantages of yeast and zebrafish ATP13A2 deficiency models, combining these two bioassay systems to create an integrated screening platform for drug repurposing. Ultimately, this platform might accelerate both the repurposing of existing drugs, as well as the discovery of new lead compounds for the treatment of neurodegenerative disorders associated with the dysregulation of heavy metal homeostasis. As a next step, both NAC and FTD, as well as some of the other hits with activity only in the yeast model, should be tested in other in vivo models of ATP13A2 deficiency, such as mouse or dog (Farias et al., 2011; Schultheis et al., 2013).

**Supplementary material**

Supplementary material is available at Brain Communications online.

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**Competing interests**

The authors report no competing interests.

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