MANUSCRIPT TITLE

Overcoming the widespread flaws in the annotation of vertebrate selenoprotein genes in public databases

AUTHORS

Max Ticó[1,2], Emerson Sullivan[3], Roderic Guigó[2] and Marco Mariotti[1*]

[1] Department of Genetics, Microbiology and Statistics, Universitat de Barcelona, Barcelona, Catalonia, 08024, Spain
[2] Centre for Genomic Regulation (CRG), The Barcelona Institute for Science and Technology (BIST), Barcelona, Catalonia, 08003, Spain.
[3] Johns Hopkins Whiting School of Engineering, Baltimore, MD, 21218, United States.
* To whom correspondence should be addressed. Email: marco.mariotti@ub.edu

ABSTRACT

Selenocysteine (Sec) is a non-canonical amino acid incorporated into selenoproteins, oxidoreductase enzymes carrying essential roles in redox homeostasis. Sec insertion is performed in response to the UGA codon, normally interpreted as a stop codon, but recoded in selenoprotein mRNAs. Owing to the dual function of UGA, the identification of selenoprotein genes poses a challenge.

We show here that the vertebrate selenoprotein genes are widely misannotated in the major public databases. In Ensembl, considered the gold standard of genomic annotation, our analysis shows that only ~10% of selenoprotein genes are well annotated; ~10% have no annotation at all, and ~80% have flawed annotations which lack the Sec-encoding UGA. Only model organisms have correct selenoprotein annotations in Ensembl, ascribed to manual curation. At NCBI, ~50% of selenoproteins are misannotated, mostly corresponding to families with C-terminal Sec residues.

We argue that selenoproteins must be correctly annotated in public database and that must occur via automated pipelines, to keep the pace with genome sequencing. To facilitate this task, we present a new version of Selenoprofiles, an homology based tool for prediction of selenoproteins that can be easily deployed and produce correct predictions with accuracy comparable to manual curation.

INTRODUCTION

Selenoproteins are a group of proteins that contain the amino acid selenocysteine (Sec or U), the twenty-first amino acid in the genetic code. Sec is co-translationally inserted via recoding of UGA, a codon which normally terminates protein synthesis (Labunskyy et al., 2014; Mariotti & Gladyshev, 2022). Sec insertion primarily depends on a *cis*- acting RNA structure called SECIS element, located in 3′ UTR regions of selenoprotein mRNAs of eukaryotes (Howard & Copeland, 2019). The human selenoproteome consist of 25 selenoprotein genes, which have important roles in various biological processes including redox homeostasis, hormone maturation, immune response, and others (Kryukov et al., 2003; Mariotti & Gladyshev, 2022). Thioredoxin reductases (TXNRD), glutathione peroxidases (GPX) and iodothyronine deiodinases (DIO) are represented by 3, 5, and 3 selenoprotein genes in human, respectively, and they are arguably the most well studied eukaryotic selenoprotein families. TXNRDs and GPXs are essential components of the two major antioxidant systems in mammals, the thioredoxin and glutathione system respectively, while DIOs control thyroid hormone maturation. The functions of approximately a third of human selenoproteins are yet uncharacterized or poorly defined. Most selenoprotein families include homologs that substitute Sec with cysteine (Cys), i.e. "Cys homologs". These may be found as orthologs (e.g. Drosophila thioredoxin reductase *Trxr1*) and/or as paralogs (e.g. human *GPX5*, *GPX7*, *GPX8*).

Selenoprotein genes have been identified in diverse branches of the tree of life. In this work, we focus on eukaryotes only. We previously described the vertebrate selenoproteome as consisting of 24-38 selenoprotein genes, based on an analysis of 44 genomes available in 2012 (Mariotti et al., 2012). Sec usage was also observed in other eukaryote species, including most metazoa, algae, some protists, and a handful of fungi (Lobanov et al., 2009; Mariotti et al., 2019; Santesmasses et al., 2020). On the other hand, Sec is absent in higher plants, yeasts, many insects and few nematodes (Chapple & Guigó, 2008; Mariotti et al., 2019; Otero et al., 2014; Santesmasses et al., 2020).

Owing to this dual function of the UGA codon, selenoproteins are usually neglected by standard gene annotation programs, so that specialized software is required (Rajput et al., 2019; Santesmasses et al., 2020). Relevantly for this work, our group previously developed Selenoprofiles, an automated pipeline to predict selenoprotein genes by homology to a set of

built-in profiles representing known selenoprotein families (Mariotti & Guigó, 2010; Santesmasses et al., 2018).

In the present work, we assessed selenoprotein gene annotation in two major public databases, Ensembl and NCBI, focusing on vertebrate genomes. By using Selenoprofiles to predict selenoproteins in genome assemblies and assessing the corresponding gene annotations, we show evidence for widespread misannotation of selenoprotein genes, particularly in Ensembl. To remedy this important issue, we present new technical advancements in Selenoprofiles, improving its accuracy and facilitating its execution and integration in annotation pipelines.

MATERIAL AND METHODS

Genomic sequences and resources

We downloaded all assemblies and annotations available in Ensembl version v.108 (Cunningham et al., 2022), comprising of 315 genomes, each corresponding to a different species or strain. For the data at the National Center for Biotechnology Information (NCBI), we use the tool "NCBI datasets" to obtain all genome assemblies corresponding to vertebrates, then considered only those with an available gene annotation (O'Leary et al., 2024; Sayers et al., 2021). Because some annotations were available but limited to the mitochondrial genome or scaffold structure, we only considered GTF files whose archive size was >3MB. This resulted in a set of 1,010 NCBI vertebrate genomes, each representing a distinct species. The phylogenetic tree of Ensembl species we downloaded from Ensembl Compara (Herrero et al., 2016). The analogous NCBI tree was obtained from NCBI Taxonomy (Schoch et al., 2020).

Selenoprotein gene prediction

Selenoprotein gene prediction was performed using Selenoprofiles (Mariotti & Guigó, 2010; Santesmasses et al., 2018) version v4.5.0, available at https://github.com/marco-mariotti/selenoprofiles4. Selenoprofiles is a computational pipeline to identify members of the known selenoprotein families and related proteins. It uses several homology-based gene prediction programs, and employs a set of manually curated multiple sequence alignments of selenoprotein families to scan genomes for homologues. The profiles we used are available at https://github.com/marco-mariotti/selenoprotein_profiles (v1.2.0). We scanned genome assemblies for all known selenoprotein families in metazoa and focused our analyses on the

predictions labelled as "selenocysteine", i.e., the genes encoding for selenoproteins, with no apparent pseudogene features (e.g. frameshifts).

Annotation assessment

To assess the annotation of selenoproteins in public databases, we developed a new utility called *Selenoprofiles assess*, now integrated in Selenoprofiles v4.5.0. This tool takes as input two GTF/GFF files, one from Selenoprofiles and one from another source, and matches gene annotations based on their overlap in genomic coordinates. After analysis of the Sec-encoding codon and the rest of coding sequence, each Selenoprofiles prediction is assigned an annotation status label, as shown in Figure 1 and described in Results. To make this script, we took advantage of PyRanges, a package to represent and manipulate genomic data in Python (Stovner & Sætrom, 2020). Plots were built using the R package ggplot2 (Wickham, 2009).

Phylogenetic reconstruction and orthology

The protein sequences of all Selenoprofiles predictions across Ensembl genomes were collected using the *Selenoprofiles join* utility. These were used as input to phylogenetic reconstruction workflows, available in the ETE3 v3.1.3 build framework (Huerta-Cepas, Serra, et al., 2016). We ultimately presents trees obtained with the "eggnog41" workflow (Huerta-Cepas, Szklarczyk, et al., 2016), which internally uses PhyML v20160115.patched for phylogenetic reconstruction (Guindon et al., 2010). We visualized gene trees with the *show_tree* program in the biotree_tools package v0.0.6 (https://github.com/marco-mariotti/biotree_tools.git), based on the ETE3 python module (Huerta-Cepas, Serra, et al., 2016).

For every selenoprotein family with multiple paralogs in vertebrates (GPX, TXNRD, DIO, SELENOW/V), we inspected phylogenetic trees and manually partitioned them into orthologous groups, here referred to as subfamilies, by applying the species overlap method and following maximum parsimony criteria (Gabaldón, 2008). To assign subfamily names, we performed similarity searches against Uniprot and consulted relevant literature (Gladyshev et al., 2016; Hirt et al., 2002; Mariotti et al., 2012).

Next, we built a new utility called *Selenoprofiles orthology*, now available in v4.5.0, constituting a fast alternative to tree building for subfamily assignment. To this aim, we first generated reference alignments ("anchors") for each multimember selenoprotein family including representatives of each subfamily, which were assigned to orthologous groups beforehand via

phylogenetic reconstruction as detailed above. Alignments were performed using Mafft v7.45331 (Katoh & Standley, 2013). Anchor alignments were reduced to a maximum of 4, 8, or 12 representative sequences for each subfamily for each lineage, automatically selected using TrimAl (Capella-Gutiérrez et al., 2009) to preserve sequence diversity. In the *Selenoprofiles orthology* procedure, candidate sequences are aligned to reference alignments using Mafft, and Pyaln v0.1.4 (https://github.com/marco-mariotti/pyaln.git) is used for fast calculation of similarity scores of candidate sequences against the representative sequences of each subfamily. These scores are used for subfamily assignment of each candidate. Similarity score parameters are displayed in Table S1. Results of our benchmark with 3,952 Selenoprofiles predictions in Ensembl genomes are shown in Table S2, which led to choosing anchor alignments with size of 12 sequences, and this Pyaln sequence comparison method: gap positions are excluded (gaps = n), and the average weighted sequence identity "AWSI" (metrics = 'w') is computed with weights based on the maximum frequency of non-gap characters (weights = 'm'). The same similarity metrics is used for ranking gene predictions in the novel filtering procedure *Selenoprofiles lineage* (see Results). Sequence and tree manipulations throughout the project were carried out using the programs *alignment_tools* and *tree_tools* from the biotree_tools package.

RESULTS

Evaluation of selenoprotein annotation

We set to evaluate the status of selenoprotein annotations in Ensembl and NCBI. The identification of all selenoprotein genes in a genome is not trivial, and it is likely that many selenoprotein families remain undiscovered in less studied organisms, e.g. unicellular eukaryotes (Santesmasses et al., 2020). To elude this problem, we focused on vertebrates: their selenoproteome has been thoroughly studied (Castellano et al., 2004, 2005; Davy & Castellano, 2018; Kryukov et al., 2003; Mariotti et al., 2012; Novoselov et al., 2006; Sarangi et al., 2018; Shchedrina et al., 2007), so that, we argue, it can be identified in its entirety by homology to known selenoprotein families. Our dataset consisted of paired genome assemblies and genome annotations, 315 from Ensembl and 1,010 from NCBI (Methods). As *bona fide* selenoprotein annotation, we ran Selenoprofiles, which yielded 8,629 and 26,210 selenoprotein predictions in Ensembl and NCBI genomes, respectively, corresponding to 21 protein families

protein families (see Data Availability). Later on, we critically evaluated and refined this method, as detailed in later sections of this manuscript.

We inferred the status of selenoprotein annotation by matching genomic coordinates from Selenoprofiles predictions with annotated coding sequence (CDS) regions and evaluating whether the Sec codon was included in the annotated CDS. If it was not the case, we further classified them into different misannotation cases depending on the position of Sec and annotated CDS (Figure 1). CDS annotations that ended precisely at the Sec UGA were classified as “Stop codon”. The misannotation classes “Upstream”, “Downstream”, and “Skipped” were applied when there were portions of annotated CDS at the 5′, 3′, or both 5′ and 3′ of the Sec UGA, respectively. Some selenoprotein annotations exhibited a distinct frame compared to Selenoprofiles predictions, and were classified as "Out of frame" cases. Transcripts which contained a splicing site in the Sec codon were classified as “Spliced”. Instances where no annotation was overlapping to a Selenoprofiles selenoprotein CDS were classified as "Absent".

The great majority of selenoprotein families contain a single Sec residue. Notable exceptions in vertebrates are SELENOP, which contains 10 Secs in human and up to 37 in fish (Baclaocos et al., 2019), and SELENOL, which contains two nearby Sec forming a diselenide bond (Shchedrina et al., 2007). While the first Sec residue of SelenoP is embedded in a conserved thioredoxin-like motif, the rest are located in a Sec-rich C-terminal tail, which is particularly challenging to identify in genomes (Baclaocos et al., 2019). For simplicity, here we considered only the first Sec residue of each selenoprotein gene to define its annotation status.

Figure 1: Classification of selenoprotein annotation/misannotations. *Segments represent coding sequence (CDS) regions of selenoprotein genes. Selenoprofiles predicted CDS are colored in dark grey, and annotated Ensembl/NCBI transcripts are in light grey. Dotted vertical lines represent start and end positions of the Sec encoding codon. Introns were shrunk to a fixed size for visualization purposes. The classes in blue, i.e. all other than "Well annotated" and "Absent", are collectively referred to with the term "Misannotation" in this manuscript.*

The status of selenoprotein annotation in Ensembl

Our results show that most selenoprotein genes have defective annotations in Ensembl: only ~10% of predicted selenoproteins present at least one "Well annotated" entry (Figure 2A). Around 10% of selenoproteins have no annotation at all ("Absent"). The majority (~80%) are "Misannotations", i.e. there are only flawed annotations which lack the Sec-encoding UGA. We next examined annotation at transcript level, wherein a single Selenoprofiles gene prediction may match multiple annotated transcripts, particularly in well studied model organisms. This analysis allowed us to distinguish among misannotation types (Figure 1). We observed that almost 25% of transcripts skip the Sec-UGA-containing exon, or at least its relevant portion (Figure 2B). There are also numerous cases where the annotated CDS ends at the Sec codon (18.3%), or further upstream (22.1%), or starts downstream of it (17.6%). Other cases were more rare.

Next, we examined the annotation status for each selenoprotein family separately. Surprisingly, the most well-known selenoprotein families do not exhibit the best annotation status in Ensembl (Figure 2C); rather, SELENOH and SELENOP (first Sec) exhibit the highest percentage of "Well annotated" cases (29.7% and 24.6%, respectively). Consistent with intuition, we observed a clear correlation between misannotation type and the position of Sec in the gene structure. In protein families that carry Sec in close proximity to the C-terminus, such as TXNRDs and selenoproteins S, O, K, and I, the Sec residue tends to be annotated as a "Stop codon" signal, or the CDS ends further "Upstream" (Figure 2E). On the other hand, "Downstream" cases are more abundant in families containing Sec in their N-terminal part, such as GPX, DIO, SPS, and selenoproteins H, M, N, T, V, W. Unsurprisingly, the selenoproteins SELENOE (Novoselov et al., 2006) and SELENOL (Shchedrina et al., 2007), that are not present in human and mouse, exhibit the worst status in Ensembl, with zero "Well

annotated" genes. Curiously, this is not the case for selenoprotein SELENOJ, despite its presence is also limited to fish (and sea urchin) genomes (Castellano et al., 2005).

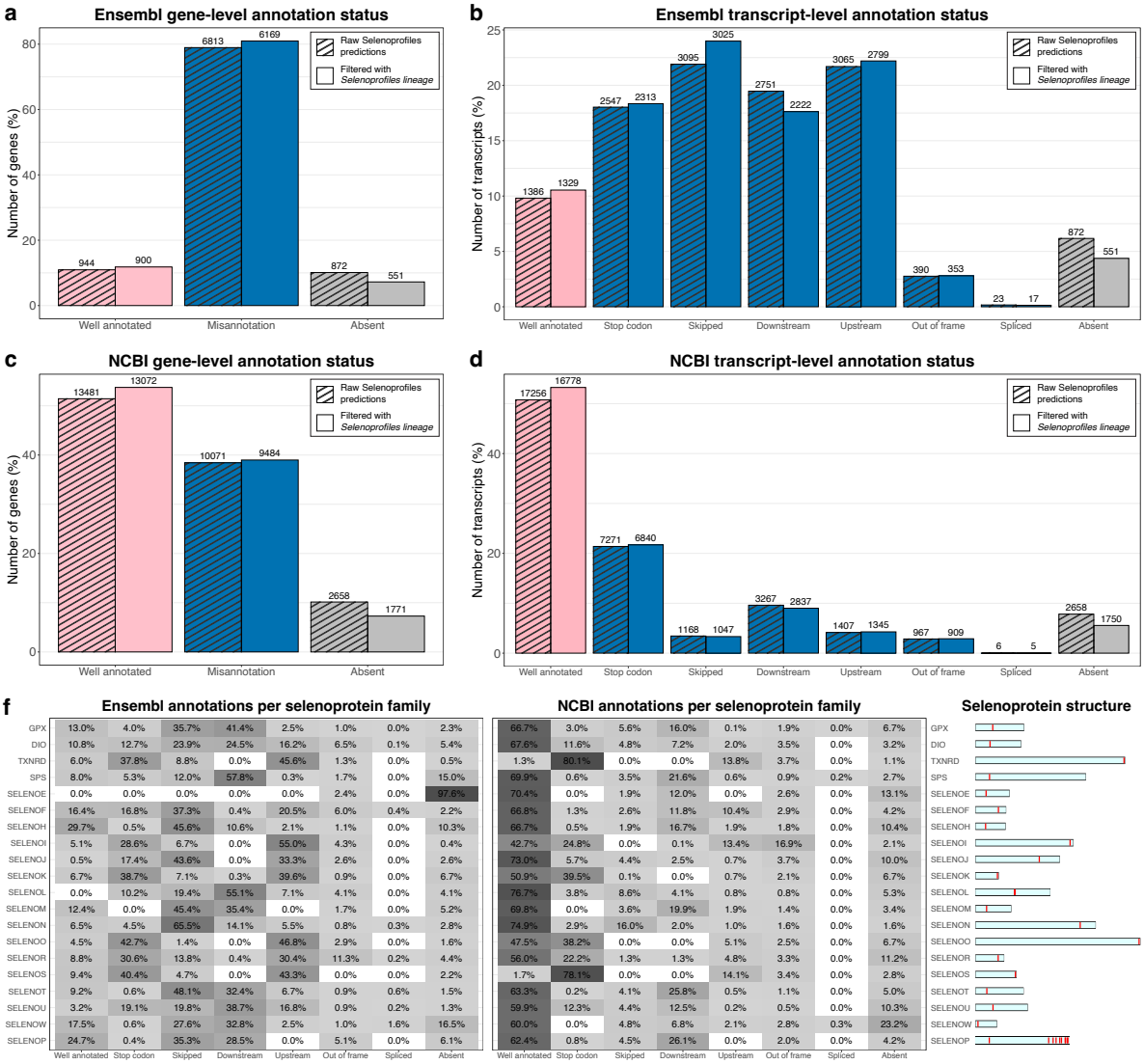

Figure 2. Status of selenoprotein annotation in Ensembl and NCBI. a. *Bar plot showing the annotation status of selenoproteins (Selenoprofiles predictions) at gene level in the Ensembl database. Three main annotation cases are shown, with "Misannotation" aggregating all classes shown in Figure 1 other than "Well annotated" and "Absent". Bar shading differentiates the raw (i.e. unfiltered) Selenoprofiles predictions and those filtered with Selenoprofiles lineage as explained later in the manuscript. Bar height represents percentages, and numbers on top of bars show actual counts.* ***b.*** *Bar plot showing the annotation status at transcript level in Ensembl, with all possible annotation types.* ***c.*** *Gene-level annotation status of selenoproteins at NCBI.* ***d.*** *Transcript-level annotation status of selenoproteins at NCBI.* ***e.*** *Heatmap representing the distribution of annotation/misannotation classes for each selenoprotein family, calculated at*

transcript level using filtered predictions, for Ensembl (left) and NCBI (center). The right panel shows the gene structures of representatives of each family, with Sec residues displayed as red lines. Note that the SELENOW contains both SelenoW and SelenoV genes, since they are highly homologous and included in the same Selenoprofiles profile.

We noticed that misannotations are not homogenously distributed across species. Instead, "Well annotated" selenoprotein genes are concentrated in few model species (e.g. human, mouse, zebrafish, pig). In contrast, almost all selenoproteins are misannotated in the rest of species. The full tree of Ensembl species with the complete classification of their selenoprotein genes is shown in Supplementary Figure S1, while Figure 3 shows a reduced version with a representative set of species. This pattern is consistent with selenoprotein genes being correctly annotated for model organisms via manual curation.

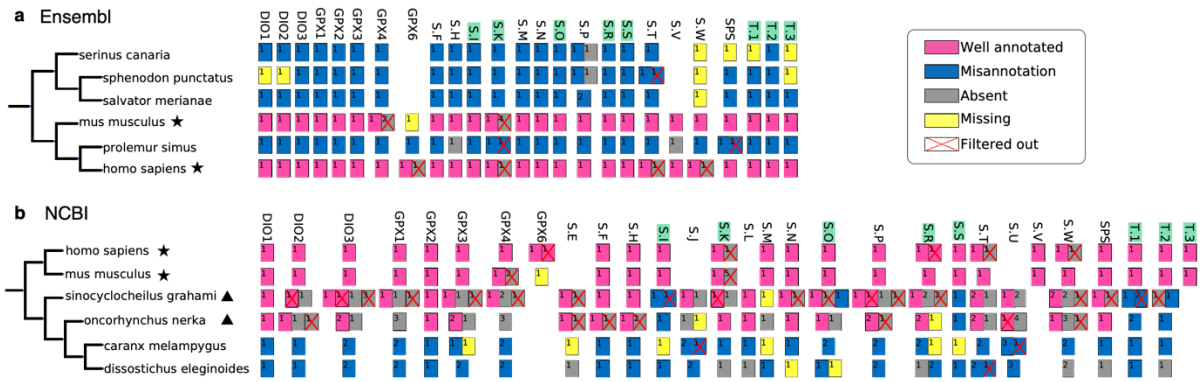

Figure 3. Distribution of misannotations across species. *The species tree of six representative organisms is shown with the annotation of selenoprotein genes in Ensembl (**a**) and NCBI (**b**), color-coded by annotation/misannotation labels grouped by subfamily. Gene-level annotation classes are used (Figure 1), wherein "Absent" (grey) means that a selenoprotein predicted by Selenoprofiles has no overlapping annotation in Ensembl/NCBI. Moreover, the yellow color is used to mark "Missing" genes, i.e. expected in this genome based on lineage but not detected by Selenoprofiles. Also, red crosses mark those genes "Filtered out" by Selenoprofiles lineage, i.e. unexpected genes. The names of selenoprotein families and subfamilies (columns) are denoted with abbreviations: "S." stands for SELENO, and "T." stands for TXNRD. Subfamilies with C-terminal Sec are highlighted in green. In the species tree, stars denote model organisms with well*

annotated selenoproteomes, and a triangle denote NCBI genomes with well annotated selenoproteins except for the C-terminal Sec families.

The status of selenoprotein annotation in NCBI

Our analysis of vertebrate NCBI genome annotations show that around half of selenoprotein genes have "Well annotated" Sec residues. The rest of cases are either "Absent" (7.3%) or "Misannotations" (38.9%). By analyzing the distribution of misannotation types at transcript-level and by family (Figure 2DE), it appears evident that the main flaw of NCBI annotation of selenoproteins concerns the selenoproteins with C-terminal Sec residues. Indeed, the "Stop codon" class constitutes more than half of "Misannotation" cases. It also appears that, in NCBI, the annotation status of fish-specific selenoprotein families is not worse than mammalian families.

Analyzing of the distribution of misannotations across species (representative tree in Figure 3; full set in Supplementary Figure S2;), we notice that we can divide NCBI genome annotations in three groups: 1. A handful of model species have selenoproteomes that are completely well annotated; 2. Some species have well annotated selenoproteins, except for the C-terminal Sec protein families; 3. The rest of species have all selenoproteins misannotated or missing. These categories were scattered across all lineages of vertebrates, with no evident pattern. We did notice, however, that birds tended to fall in the third category, therefore making up the bulk of NCBI misannotations.

Improving Selenoprofiles for fully automated reliable annotation of selenoproteins

We set to provide an fully automated computational tool to remedy the poor status of selenoprotein annotation in public databases. Previously, we developed Selenoprofiles, a pipeline for selenoprotein gene prediction in genomes featuring remarkable gene prediction accuracy (Mariotti & Guigó, 2010). Yet, Selenoprofiles was not integrated in the annotation pipeline of public databases, to our knowledge. We ascribe this at least partially to the characteristics of the early versions of Selenoprofiles, which were hard to install and run. Here, we present a new version which remedied these shortcomings and introduce various other improvements. First, Selenoprofiles can now be installed via a single conda command, or by pulling a ready-made Docker image, as explained in the new comprehensive online documentation (https://selenoprofiles4.readthedocs.io/).

***Selenoprofiles orthology*: automatic assignment of selenoprotein subfamilies**

Second, we created a new utility called *Selenoprofiles orthology* to assign gene predictions to specific subfamilies, i.e. orthologous groups / paralogs. Indeed, Selenoprofiles predicts all paralogous genes of a given family (e.g. DIO1, DIO2, DIO3) using the same profile (DIO), so that, in previous versions, the user could not differentiate among paralogs without a dedicated posterior analysis. The *Selenoprofiles orthology* utility addresses this task by adopting a fast alternative to phylogenetic tree reconstruction, the state-of-the-art for orthology assignment (Figure 4). Our procedure is based on curated reference alignments referred to as "anchors", containing selected representatives for all subfamilies for each multimember selenoprotein family. New selenoprotein predictions are aligned to anchors, and their protein sequence similarity is computed against each subfamily, ultimately assigning each prediction to its most similar subfamily.

To develop this functionality, we built gene trees and manually partitioned them into orthologous groups (Methods), which replicated well our previous phylogenetic analysis of vertebrate selenoproteins (Mariotti et al., 2012). From these, we derived anchor alignments with reliable subfamily labels, which we then reduced for faster processing by automatic selection of sequence representatives. For subfamily assignment of candidates, we tested various metrics of sequence similarity, differing by how gaps are scored and how different columns are aggregated. We then selected the method that maximized the accuracy of orthology assignment in a set of 3,952 selenoprotein predictions from four multimember families (GPX, TXNRD, DIO and SELENOW/V) in Ensembl genomes (Methods; Supplementary Tables S1 and S2).

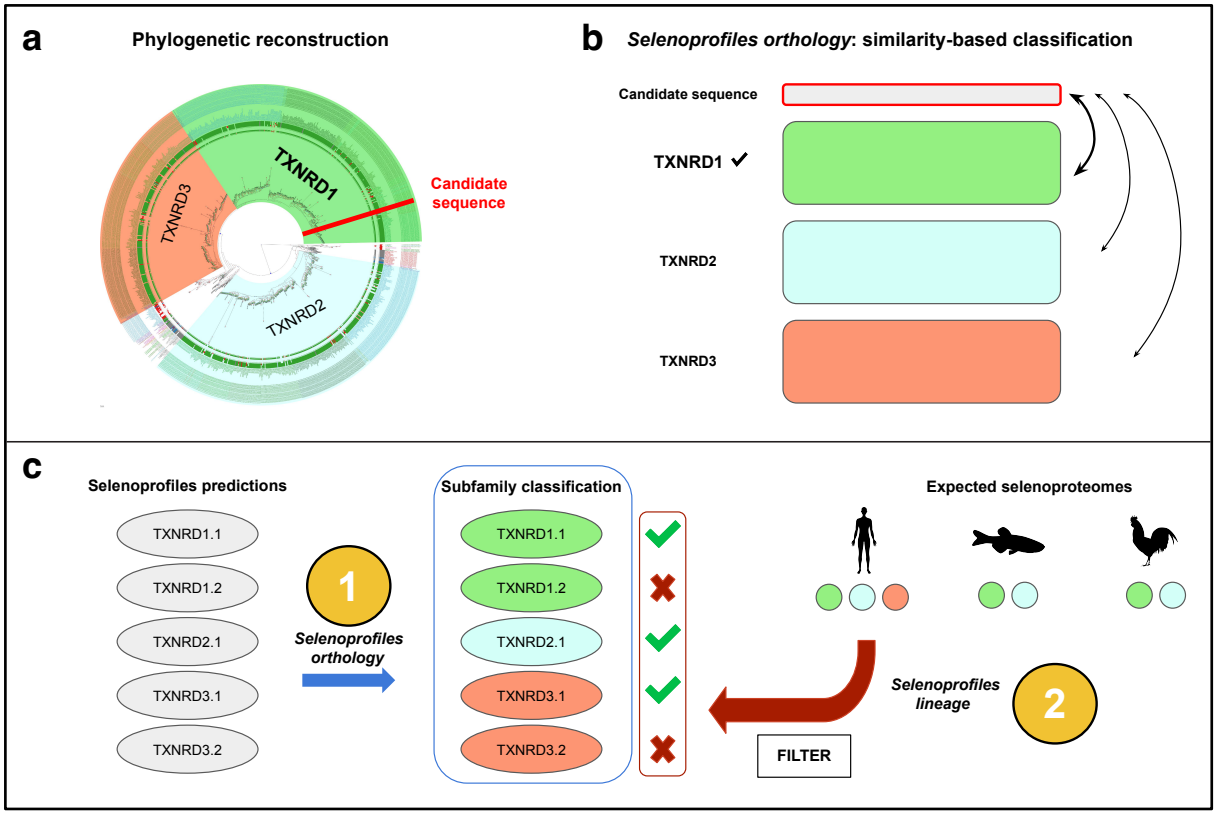

Figure 4. New Selenoprofiles utilities for subfamily classification pipeline and prediction refinement. *Initially, we built a phylogenetic tree for every multimember family to produce a bona-fide classification of orthologous groups (**a**). Next, we devised an automated, fast similarity-based procedure that does not require tree building, now implemented as the Selenoprofiles orthology utility (**b**). Finally, we developed a filter based on the expected selenoproteome for each lineage, now implemented as the Selenoprofiles lineage utility (**c**).*

***Selenoprofiles lineage*: filtering based on expected selenoproteomes per lineage**

Lastly, we decided to improve Selenoprofiles so that its predictions roughly match the accuracy of experienced manual curation. While our pipeline already features high sensitivity, it can lead to some false positives, typically in presence of abundant retrotransposed pseudogenes in mammalian genomes. In Selenoprofiles, most pseudogenes are already labelled and discarded due to the presence of in-frame stop codons or frameshifts, but some happen to lack these features and are output. At manual inspection, these may be identified by three criteria: 1. they are typically intronless, 2. they lack evidence of transcription, and 3. they are not conserved. Because some functional genes are also intronless (e.g. Sephs2 (Mariotti et al., 2012, 2015)), the first criterion is not fit for inclusion in an automated pipeline. The second criterion is also not suitable for Selenoprofiles, because it requires additional data (transcriptomics) not

universally available. We thus decided to implement the conservation criteria in a novel utility called *Selenoprofiles lineage*, which consists in a filter based on the set of selenoproteins previously observed in other species related to the one under analysis. This functionality depend on a manually curated table defining the precise selenoprotein content of all major vertebrate lineages (Data Availability). We compiled this data based on our previous analysis of vertebrate selenoproteomes (Mariotti et al., 2012), now replicated and expanded to 315 Ensembl genomes.

Selenoprofiles lineage (Figure 4C) is built on top of the *orthology* utility: gene expectations are defined at the subfamily level, for improved precision. First, the species under analysis is mapped into a vertebrate lineage present in the expectation table (e.g. placentals, birds), either manually or automatically via NCBI taxonomy. Next, the program checks for extra predicted genes: for example, two copies of GPX4 are found in the mouse genome, but a single one is expected in placentals (Figure 3). Finally, genes are selected based on sequence similarity to the subfamily anchor alignment: only the N top-scoring candidates are kept, where N is the number of expected genes. Besides marking some genes as "Filtered out", *Selenoprofiles lineage* may also report some subfamilies as "Missing", if a given genome contains fewer predictions than expected.

Our new utility allow to refine predicted selenoproteomes in full automation. Results are shown in Figure 1, which shows the annotation status of Ensembl and NCBI before and after lineage-based filtering, and in Figure 3, and Supplementary Figures S1 and S2, wherein filtered and missing genes are shown for each species. Mostly, filtered out predictions corresponded to "Absent" and "Misannotation" rather than "Well annotated" cases, consistent with the notion that pseudogenes are carefully annotated less often than functional genes.

The new *orthology* and *lineage* functionalities are available in Selenoprofiles v4.5.0. In the future, we may expand their use to non-vertebrate genomes.

DISCUSSION

Despite the massive advances in sequencing, genome annotation remains challenging. Due to the recoding of the UGA codon, this problem is aggravated in the case of selenoprotein genes, leading to mispredictions and omissions in public databases. The current study confirmed clear support to this hypothesis: we report that the extent of flawed annotations for selenoprotein

genes reach 90% for Ensembl, and 50% for NCBI. In most cases, an overlapping gene annotation is present but does not include the Sec codon, with many possible gene structure arrangements observed (Figures 1 and 2). At NCBI, most misannotations concern selenoprotein genes with Sec residues in their C-terminal tail, which are arguably the hardest to reliably identify by any method. Importantly, large differences were observed between model and non-model organisms. Well-studied organisms like human, mouse, and zebrafish have typically manually curated genome annotations wherein selenoproteins may have been corrected. Indeed, NCBI previously reported the manual annotation of selenoproteins in 9 vertebrate genomes (Rajput et al., 2019).

Genome annotations are essential for understanding evolutionary relationships, molecular functions, gene expression and regulation, among others, and non-model organisms in particular are subject of unprecedented scientific attention. Indeed, we entered an era of massive sequencing and biodiversity genomics, and in the near future the goal is to sequence all eukaryotic life on Earth, driven by initiatives under the umbrella of the Earth Biogenome Project (Corominas et al., 2024; Lewin et al., 2018; Mc Cartney et al., 2024). Naturally, manual curation cannot keep the pace of modern genomics, and having accurate automated pipelines is compelling.

It is now more important than ever that genome annotations of all organisms are as complete as possible, to enable the effective application of methodologies that make use of annotations as a key source of information. This includes a wide array of omics technologies, phylogenetic studies, and also unbiased experimental and computational approaches such as CRISPR-based screening for genes involved in a pathway of interest, and genome scans for positive selection. In these contexts, omissions and misannotations may lead to biases, incomplete design, and misguided interpretation of results.

One may think that Sec residues represent only a tiny fraction of our genomes, and may unwisely dismiss their importance. Yet, selenoprotein genes are responsible for many essential functions: most notably, they are core components of the antioxidant and redox homeostasis systems of all vertebrates, which are fundamental for life. In these enzymes, Sec is located almost invariantly in the catalytic site, where it provides a selective advantage over its canonical analog cysteine, which may be related to increased resistance to oxidative inactivation, or

enhanced catalysis (Mariotti & Gladyshev, 2022). Therefore, Sec is arguably the most important residue of many essential proteins, and their correct annotation is compelling.

In previous attempts to address the issue of selenoprotein prediction, a dedicated database was created, called SelenoDB (Castellano et al., 2008; Romagné et al., 2014), now hosted at http://selenodb.crg.eu/. Yet, we reckon that a major flaw of this approach is that, in practice, only experts of selenium biology or translational recoding typically use this database. This leaves the great majority of the scientific community, which overwhelmingly uses public annotation databases, unaware and ill-equipped towards this issue. We argue that the only possible effective strategy is to correct selenoproteins in the public annotations that researchers are already using, such as Ensembl and NCBI.

To facilitate this task, we developed a new version of Selenoprofiles, a pipeline that we already showed as effective for homology-based prediction of selenoprotein in genomes (Mariotti & Guigó, 2010). Selenoprofiles is now straightforward to install and run, it has a new extensive online documentation, and features new functionalities: assignment of subfamilies (i.e. precise gene naming) and lineage-based filtering, which improved specificity to a level comparable to manual curation. These are implemented for vertebrates, acting as straightforward test cases, and may be eventually extended to other eukaryotic lineages.

We argue and advocate for the integration of Selenoprofiles in the annotation pipelines used for public databases. We reckon that misannotations are mainly due to the in-frame UGA being penalized in the algorithms used, leading to alternative Sec-lacking gene models being selected (Figure 5). Therefore, we suggest that the output by Selenoprofiles should be fed to the combiner module that ultimately generate gene models in annotation pipelines, as an additional information layer besides the genomic data already used (e.g. for Ensembl, GeneWise predictions using Uniprot proteins and Exonerate mapping of available cDNAs)(Aken et al., 2016). Selenoprofiles only interrogates the genome sequence, while annotation pipelines typically also use transcriptomic information, so that Selenoprofiles predictions may be less accurate in regions far from Sec residue. We thus recommend that an optimal strategy would be to integrate in public annotation pipelines only the Sec encoding codons output by Selenoprofiles, prioritizing them as a high-score layer to build gene models (Figure 5).

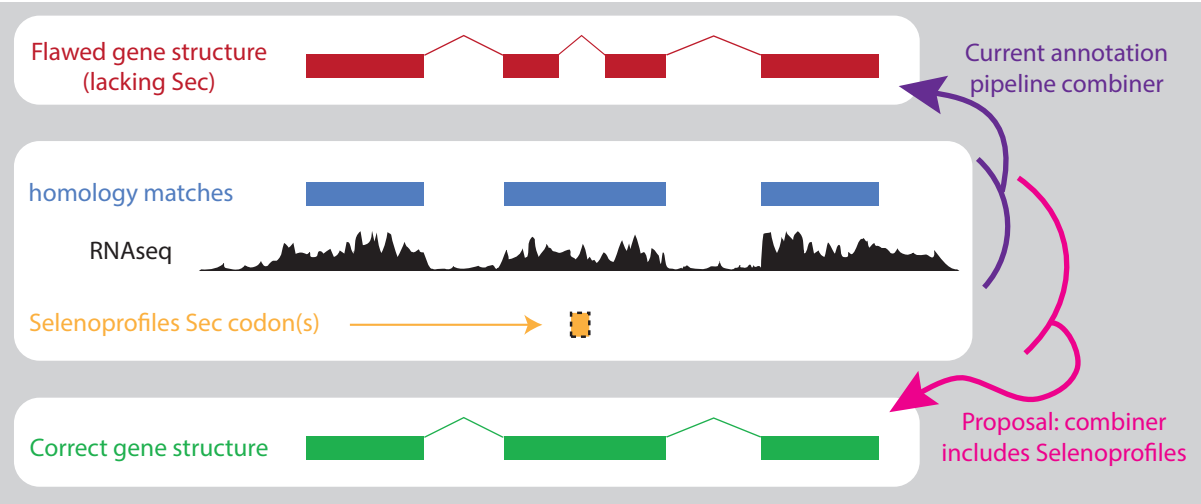

Figure 5. Proposed integration of Selenoprofiles in gene annotation pipelines. *While existing pipelines differ in their structure, all follow the same principle in that various information layers are ultimately combined into gene structures. We propose to integrate Sec codons predicted by Selenoprofiles as high-scored layer to correct selenoprotein annotation.*

Having created scalable computational tools suitable for correct annotation of selenoproteins in genomes, we will advocate for their use in public databases. We also argue that, in the near future, other well characterized cases of translational recoding (Rodnina et al., 2019) should receive a similar attention.

DATA AVAILABILITY

The latest Selenoprofiles code is available at https://github.com/marco-mariotti/selenoprofiles4 and documented at https://selenoprofiles4.readthedocs.io/. Profile alignments, anchor alignments, and expectation tables of selenoproteins per lineage are provided as companion package https://github.com/marco-mariotti/selenoprotein_profiles. Selenoprofiles predictions on Ensembl and NCBI genomes, and related annotation status labels, can be found at https://mariottigenomicslab.bio.ub.edu/selenoprofiles_data/selenoprofiles_predictions/.

Supplementary Data are available online.

AUTHOR CONTRIBUTIONS

Max Ticó: Data curation, Formal analysis, Methodology, Visualization, Writing – original draft. Emerson Sullivan: Formal analysis. Marco Mariotti: Conceptualization, Methodology, Writing—

review & editing, Supervision, Funding acquisition. Roderic Guigó: Conceptualization, Writing— review & editing.

FUNDING

Grants RYC2019-027746-I and PID2020-115122GA-I00 funded by MICIU/AEI (Ministry of Science, Innovation and Universities; State Research Agency) /10.13039/501100011033 and "ESF Investing in your future".

CONFLICT OF INTEREST

Authors declare no conflict of interest.

REFERENCES

Aken, B. L., Ayling, S., Barrell, D., Clarke, L., Curwen, V., Fairley, S., Fernandez Banet, J., Billis, K., García Girón, C., Hourlier, T., Howe, K., Kähäri, A., Kokocinski, F., Martin, F. J., Murphy, D. N., Nag, R., Ruffier, M., Schuster, M., Tang, Y. A., … Searle, S. M. J. (2016). The Ensembl gene annotation system. *Database: The Journal of Biological Databases and Curation*, *2016*, baw093. https://doi.org/10.1093/DATABASE/BAW093

Baclaocos, J., Santesmasses, D., Mariotti, M., Bierła, K., Vetick, M. B., Lynch, S., McAllen, R., Mackrill, J. J., Loughran, G., Guigó, R., Szpunar, J., Copeland, P. R., Gladyshev, V. N., & Atkins, J. F. (2019). Processive Recoding and Metazoan Evolution of Selenoprotein P: Up to 132 UGAs in Molluscs. *Journal of Molecular Biology*, *431*(22), 4381–4407. https://doi.org/10.1016/j.jmb.2019.08.007

Capella-Gutiérrez, S., Silla-Martínez, J. M., & Gabaldón, T. (2009). trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics (Oxford, England)*, *25*(15), 1972–1973. https://doi.org/10.1093/bioinformatics/btp348

Castellano, S., Gladyshev, V. N., Guigo, R., & Berry, M. J. (2008). SelenoDB 1.0: a database of selenoprotein genes, proteins and SECIS elements. *Nucleic Acids Research*, *36*(Database issue), D332-8. http://nar.oxfordjournals.org/cgi/content/abstract/gkm731v1

Castellano, S., Lobanov, A. V. A. V, Chapple, C., Novoselov, S. V. S. V., Albrecht, M., Hua, D., Lescure, A., Lengauer, T., Krol, A., Gladyshev, V. N. V. N., Guigó, R., & others. (2005). Diversity and functional plasticity of eukaryotic selenoproteins: identification and characterization of the SelJ family. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(45), 16188. https://doi.org/10.1073/pnas.0505146102

Castellano, S., Novoselov, S. V, Kryukov, G. V, Lescure, A., Blanco, E., Krol, A., Gladyshev, V. N., & Guigó, R. (2004). Reconsidering the evolution of eukaryotic selenoproteins: a novel nonmammalian family with scattered phylogenetic distribution. *EMBO Reports*, *5*(1), 71–77. https://doi.org/10.1038/sj.embor.7400036

Chapple, C. E., & Guigó, R. (2008). Relaxation of selective constraints causes independent selenoprotein extinction in insect genomes. *PLoS ONE*, *3*. https://doi.org/10.1371/journal.pone.0002968

Corominas, M., Marquès-Bonet, T., Arnedo, M. A., Bayés, M., Belmonte, J., Escrivà, H., Fernández, R., Gabaldón, T., Garnatje, T., Germain, J., Niell, M., Palero, F., Pons, J., Puigdomènech, P., Cuevas-Caballé, C., Obiol, J. F., Gut, I., Gut, M., Hidalgo, O., … Guigó, R. (2024). The Catalan initiative for the Earth BioGenome Project: contributing local data to global biodiversity genomics. *NAR Genomics and Bioinformatics*, *6*(3). https://doi.org/10.1093/NARGAB/LQAE075

Cunningham, F., Allen, J. E., Allen, J., Alvarez-Jarreta, J., Amode, M. R., Armean, I. M., Austine-Orimoloye, O., Azov, A. G., Barnes, I., Bennett, R., Berry, A., Bhai, J., Bignell, A., Billis, K., Boddu, S., Brooks, L., Charkhchi, M., Cummins, C., Da Rin Fioretto, L., … Flicek, P. (2022). Ensembl 2022. *Nucleic Acids Research*, *50*(D1), D988–D995. https://doi.org/10.1093/NAR/GKAB1049

Davy, T., & Castellano, S. (2018). The genomics of selenium: Its past, present and future. In *Biochimica et Biophysica Acta - General Subjects* (Vol. 1862, Issue 11, pp. 2427–2432). Elsevier B.V. https://doi.org/10.1016/j.bbagen.2018.05.020

Gabaldón, T. (2008). Large-scale assignment of orthology: back to phylogenetics? *Genome Biology*, *9*(10), 235. https://doi.org/10.1186/GB-2008-9-10-235/FIGURES/2

Gladyshev, V. N., Arnér, E. S., Berry, M. J., Regina, B. F., Bruford, E. A., Burk, R. F., Carlson, B. A., Castellano, S., Chavatte, L., Conrad, M., Copeland, P. R., Diamond, A. M., Driscoll, D. M., Ferreiro, A., Flohé, L., Green, F. R., Guigó, R., Handy, D. E., Hatfield, D. L., … Zhang, Y. (2016). Selenoprotein gene nomenclature. *Journal of Biological Chemistry*, *291*(46), 24036–24040. https://doi.org/10.1074/jbc.M116.756155

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., & Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology*, *59*(3), 307–321. https://doi.org/10.1093/sysbio/syq010

Herrero, J., Muffato, M., Beal, K., Fitzgerald, S., Gordon, L., Pignatelli, M., Vilella, A. J., Searle, S. M. J., Amode, R., Brent, S., Spooner, W., Kulesha, E., Yates, A., & Flicek, P. (2016). Ensembl comparative genomics resources. *Database: The Journal of Biological Databases and Curation*, *2016*. https://doi.org/10.1093/DATABASE/BAV096

Hirt, R. P., Müller, S., Martin Embley, T., & Coombs, G. H. (2002). The diversity and evolution of thioredoxin reductase: New perspectives. *Trends in Parasitology*, *18*(7), 302–308. https://doi.org/10.1016/S1471-4922(02)02293-6

Howard, M. T., & Copeland, P. R. (2019). New Directions for Understanding the Codon Redefinition Required for Selenocysteine Incorporation. *Biological Trace Element Research*, *192*(1), 18–25. https://doi.org/10.1007/s12011-019-01827-y

Huerta-Cepas, J., Serra, F., & Bork, P. (2016). ETE 3: Reconstruction, Analysis, and Visualization of Phylogenomic Data. *Molecular Biology and Evolution*, *33*(6), 1635–1638. https://doi.org/10.1093/molbev/msw046

Huerta-Cepas, J., Szklarczyk, D., Forslund, K., Cook, H., Heller, D., Walter, M. C., Rattei, T., Mende, D. R., Sunagawa, S., Kuhn, M., Jensen, L. J., von Mering, C., & Bork, P. (2016). eggNOG 4.5: a hierarchical orthology framework with improved functional annotations

for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Research*, *44*(D1), D286-93. https://doi.org/10.1093/nar/gkv1248

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution*, *30*(4), 772–780. https://doi.org/10.1093/molbev/mst010

Kryukov, G. V., Castellano, S., Novoselov, S. V., Lobanov, A. V., Zehtab, O., Guigo, R., & Gladyshev, V. N. (2003). Characterization of mammalian selenoproteomes. *Science*, *300*(5624), 1439. http://www.sciencemag.org/cgi/content/abstract/sci;300/5624/1439

Labunskyy, V. M., Hatfield, D. L., & Gladyshev, V. N. (2014). Selenoproteins: molecular pathways and physiological roles. *Physiological Reviews*, *94*(3), 739–777. https://doi.org/10.1152/physrev.00039.2013

Lewin, H. A., Robinson, G. E., Kress, W. J., Baker, W. J., Coddington, J., Crandall, K. A., Durbin, R., Edwards, S. V., Forest, F., Gilbert, M. T. P., Goldstein, M. M., Grigoriev, I. V., Hackett, K. J., Haussler, D., Jarvis, E. D., Johnson, W. E., Patrinos, A., Richards, S., Castilla-Rubio, J. C., … Zhang, G. (2018). Earth BioGenome Project: Sequencing life for the future of life. *Proceedings of the National Academy of Sciences*, *115*(17), 4325–4333. https://doi.org/10.1073/PNAS.1720115115

Lobanov, A. V, Hatfield, D. L., & Gladyshev, V. N. (2009). Eukaryotic selenoproteins and selenoproteomes. *Biochimica et Biophysica Acta*, *1790*(11), 1424–1428. https://doi.org/10.1016/j.bbagen.2009.05.014

Mariotti, M., & Gladyshev, V. N. (2022). Selenocysteine-containing proteins. *Redox Chemistry and Biology of Thiols*, 405–421. https://doi.org/10.1016/B978-0-323-90219-9.00012-1

Mariotti, M., & Guigó, R. (2010). Selenoprofiles: profile-based scanning of eukaryotic genome sequences for selenoprotein genes. *Bioinformatics*, *26*(21), 2656. https://doi.org/10.1093/BIOINFORMATICS/BTQ516

Mariotti, M., Ridge, P. G., Zhang, Y., Lobanov, A. V., Pringle, T. H., Guigo, R., Hatfield, D. L., & Gladyshev, V. N. (2012). Composition and evolution of the vertebrate and mammalian selenoproteomes. *PLoS ONE*, *7*(3). https://doi.org/10.1371/journal.pone.0033066

Mariotti, M., Salinas, G., Gabaldón, T., & Gladyshev, V. N. (2019). Utilization of selenocysteine in early-branching fungal phyla. In *Nature Microbiology* (Vol. 4, Issue 5, pp. 759–765). https://doi.org/10.1038/s41564-018-0354-9

Mariotti, M., Santesmasses, D., Capella-Gutierrez, S., Mateo, A., Arnan, C., Johnson, R., D'Aniello, S., Yim, S. H., Gladyshev, V. N., Serras, F., Corominas, M., Gabaldón, T., & Guigó, R. (2015). Evolution of selenophosphate synthetases: emergence and relocation

of function through independent duplications and recurrent subfunctionalization. *Genome Research*, *25*(9), 1256–1267. https://doi.org/10.1101/gr.190538.115

Mc Cartney, A. M., Formenti, G., Mouton, A., De Panis, D., Marins, L. S., Leitão, H. G., Diedericks, G., Kirangwa, J., Morselli, M., Salces-Ortiz, J., Escudero, N., Iannucci, A., Natali, C., Svardal, H., Fernández, R., De Pooter, T., Joris, G., Strazisar, M., Wood, J. M. D., … Mazzoni, C. J. (2024). The European Reference Genome Atlas: piloting a decentralised approach to equitable biodiversity genomics. *Npj Biodiversity*, *3*(1), 28. https://doi.org/10.1038/S44185-024-00054-6

Novoselov, S. V., Hua, D., Lobanov, A. V., & Gladyshev, V. N. (2006). Identification and characterization of Fep15, a new selenocysteine-containing member of the Sep15 protein family. *Biochemical Journal*, *394*(Pt 3), 575. https://doi.org/10.1042/BJ20051569

O'Leary, N. A., Cox, E., Holmes, J. B., Anderson, W. R., Falk, R., Hem, V., Tsuchiya, M. T. N., Schuler, G. D., Zhang, X., Torcivia, J., Ketter, A., Breen, L., Cothran, J., Bajwa, H., Tinne, J., Meric, P. A., Hlavina, W., & Schneider, V. A. (2024). Exploring and retrieving sequence and metadata for species across the tree of life with NCBI Datasets. *Scientific Data 2024 11:1*, *11*(1), 1–10. https://doi.org/10.1038/s41597-024-03571-y

Otero, L., Romanelli-Cedrez, L., Turanov, A. A., Gladyshev, V. N., Miranda-Vizuete, A., & Salinas, G. (2014). Adjustments, extinction, and remains of selenocysteine incorporation machinery in the nematode lineage. *RNA (New York, N.Y.)*, *20*(7), 1023–1034. https://doi.org/10.1261/rna.043877.113

Rajput, B., Pruitt, K. D., & Murphy, T. D. (2019). RefSeq curation and annotation of stop codon recoding in vertebrates. *Nucleic Acids Research*, *47*(2), 594–606. https://doi.org/10.1093/nar/gky1234

Rodnina, M. V, Korniy, N., Klimova, M., Karki, P., Peng, B.-Z., Senyushkina, T., Belardinelli, R., Maracci, C., Wohlgemuth, I., Samatova, E., & Peske, F. (2019). Translational recoding: canonical translation mechanisms reinterpreted. *Nucleic Acids Research*. https://doi.org/10.1093/nar/gkz783

Romagné, F., Santesmasses, D., White, L., Sarangi, G. K., Mariotti, M., Hübler, R., Weihmann, A., Parra, G., Gladyshev, V. N., Guigó, R., & Castellano, S. (2014). SelenoDB 2.0: Annotation of selenoprotein genes in animals and their genetic diversity in humans. *Nucleic Acids Research*, *42*(D1). https://doi.org/10.1093/nar/gkt1045

Santesmasses, D., Mariotti, M., & Gladyshev, V. N. (2020). Bioinformatics of Selenoproteins. In *Antioxidants and Redox Signaling* (Vol. 33, Issue 7, pp. 525–536). Mary Ann Liebert Inc. https://doi.org/10.1089/ars.2020.8044

Santesmasses, D., Mariotti, M., & Guigó, R. (2018). Selenoprofiles: A computational pipeline for annotation of selenoproteins. In *Methods in Molecular Biology* (Vol. 1661). https://doi.org/10.1007/978-1-4939-7258-6_2

Sarangi, G. K., Romagne, F., & Castellano, S. (2018). Distinct patterns of selection in selenium-dependent genes between land and aquatic vertebrates. *Molecular Biology and Evolution*, *35*(7), 1744–1756. https://doi.org/10.1093/molbev/msy070

Sayers, E. W., Beck, J., Bolton, E. E., Bourexis, D., Brister, J. R., Canese, K., Comeau, D. C., Funk, K., Kim, S., Klimke, W., Marchler-Bauer, A., Landrum, M., Lathrop, S., Lu, Z., Madden, T. L., O'Leary, N., Phan, L., Rangwala, S. H., Schneider, V. A., … Sherry, S. T. (2021). Database resources of the National Center for Biotechnology Information. *Nucleic Acids Research*, *49*(D1), D10–D17. https://doi.org/10.1093/NAR/GKAA892

Schoch, C. L., Ciufo, S., Domrachev, M., Hotton, C. L., Kannan, S., Khovanskaya, R., Leipe, D., McVeigh, R., O'Neill, K., Robbertse, B., Sharma, S., Soussov, V., Sullivan, J. P., Sun, L., Turner, S., & Karsch-Mizrachi, I. (2020). NCBI Taxonomy: a comprehensive update on curation, resources and tools. *Database : The Journal of Biological Databases and Curation*, *2020*. https://doi.org/10.1093/DATABASE/BAAA062

Shchedrina, V., Novoselov, S., Malinouski, M. Y., & Gladyshev, V. N. (2007). Identification and characterization of a selenoprotein family containing a diselenide bond in a redox motif. *Proceedings of the National Academy of Sciences*, *104*(35), 13919. http://www.pnas.org/content/104/35/13919.abstract

Stovner, E. B., & Sætrom, P. (2020). PyRanges: efficient comparison of genomic intervals in Python. *Bioinformatics*, *36*(3), 918–919. https://doi.org/10.1093/BIOINFORMATICS/BTZ615

Wickham, H. (2009). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. http://ggplot2.org