Dark Proteome Database: Studies on Dark Proteins

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Abstract: The dark proteome as we define it, is the part of the proteome where 3D structure has not been observed either by homology modeling or by experimental characterization in the protein universe. From the 550.116 proteins available in Swiss-Prot (as of July 2016) 43.2% of the Eukarya universe and 49.2% of the Virus universe are part of the dark proteome. In Bacteria and Archaea, the percentage of the dark proteome presence is significantly less, with 12.6% and 13.3% respectively. In this work, we present the map of the dark proteome in Human and in other model organisms. The most significant result is that around 40%- 50% of the proteome of these organisms are still in the dark, where the higher percentages belong to higher eukaryotes (mouse and human organisms). Due to the amount of darkness present in the human organism being more than 50%, deeper studies were made, including the identification of 'dark' genes that are responsible for the production of the so-called dark proteins, as well as, the identification of the 'dark' organs where dark proteins are over represented, namely heart, cervical mucosa and natural killer cells. This is a step forward in the direction of the human dark proteome.

Keywords: Dark Proteome, Molecular Structure, Homology Modelling.

1. Introduction

Many key insights and discoveries in the life sciences have been derived from atomic-scale 3D structures of proteins. Thanks to steady improvements in experimental structure determination methods, the PDB, or Protein Data Bank (1), which stores these structures recently went past 125,000 entries - a landmark in our understanding of the molecular processes of life. This lags far behind the growth of protein sequence information, with less than 0.1% of UniProt (2) proteins linked to a PDB (1) structure. However, the understanding that evolution conserves structure more than sequence has led to large-scale computation of structural model efforts (3). Recently, we created Aquaria (4), a new resource built upon a systematic all-against-all comparison of Swiss-Prot (2) and PDB sequences, resulting in 46 million template models (4); this provides a depth of sequence-to-structure information currently not available from other resources to visible proteome. However, there is a dark side of the proteome which we were the first to categorize (5), i.e., regions of protein sequence, or whole sequences, that remain stubbornly inaccessible to either experimental structure determination or modelling approaches such as Aquaria and others. However, this knowledge was updated and is kept in the Dark Proteome Database (DPD) (6) completely independent from Aquaria (4) since our initial work where we had coined the term Dark Proteome (5) as a synonym for the structurally unknown part of the protein sequence universe, i.e., full sequences and/or regions of sequences for which structure is currently undetermined (5,6). After another definition for Dark Proteome appeared as a synonym of intrinsically disordered proteins (IDP) and/or regions (IDR) (7) narrowing the dark proteome definition down to the intrinsically unstructured proteins and/or regions, where their structure determination by conventional methods such as X-ray, Nuclear Magnetic Resonance (NMR) crystallography and Electron Microscopy (EM) are arduous, due to the failure of homology methods, but most important due to their restless nature. Besides the fact that both definitions are focused on the structurally undetermined protein sequences, the first definition is broader and wider while the later narrows it to a small part of the former, i.e., to the intrinsically unstructured regions. Nevertheless, both research paths stress the importance in studying the Dark Proteome since it is largely incomprehensible.

The present study serves as introduction for two new features that were added to the Dark Proteome Database: (i) the availability of the 'Features' (FT) field of Swiss-Prot to deepen the knowledge of dark and non-dark proteins allowing the characterization of life domains and model organisms proceed into a more thorough analysis; and (ii) the availability of an autonomy value per protein, allowing the analysis of the level of autonomy of dark and non-dark proteomes of those organisms as a whole.

Therefore, the main reasons of this work are: (i) detail even more the dark proteins present in the four domains of life, model organisms and Homo Sapiens and (ii) to map how much of darkness is still present in these model organisms, since they are so important for Mankind, i.e., how much do we know and do not know about a certain organism just by looking at the amount of darkness that it holds? Due to the number of existing organisms, we had to select the most important 'model' organisms while the ones remaining are work in progress. This choice is not innocent since sequences prevent from non-model organisms are known by the lack of sequence annotation, without being true orphan sequences. In DPD (6) we started with information from the Homo Sapiens because it is of direct concern to us, but Earth is the home for millions of organisms and only approximately two handfuls of them were adopted as "model" organisms for biological experiments, concerning food, infirmities, diseases and threats. In an ideal world, we would have access to all the knowledge concerning every single organism that inhabits this planet. However, we are not in the ideal world, and therefore these models are selected according with our present reality. Therefore, studying all of them in depth with today's technology is impossible because experimentation is complex, time consuming and highly expensive.

Keeping in mind our reality and to thin the paths of knowledge, we have to remember that some of the most valuable methods in biological research are invasive or require organism's death. For all the previous reasons and more, much of this work is impossible or unethical to perform on humans and in certain organisms. As solution, biologists have selected some model organisms to be used as testers.

The following list contains a simple plant, a worm, a bacterium or prokaryotic, a simple eukaryotic, and a complex eukaryotic or mammal. Finally, we will analyze the Human organism (Homo Sapiens) in much more detail including genes from where dark proteins come ('dark' genes) and tissues where dark proteins are expressed ('dark' tissues).

Arabidopsis thaliana (Plant)

Arabidopsis thaliana is considered a weed, being also known as mouse-ear cress and is the most widely used plant as model organism. One reason *Arabidopsis* makes a good model is because it undergoes the same exact processes of growth, flowering and reproduction as most complex plants, taking only about one month and a half to grow completely producing a huge quantity of seeds in the process. Another reason is due to the fact that *Arabidopsis* has one of the smallest genomes in the plant kingdom with only 135 mega base pairs and five diploid chromosomes, becoming the first plant with a completely sequenced genome.

Caenorhabditis Elegans (Worm)

Caenorhabditis Elegans is a soil worm or nematode and is considered a model for multicellular organisms. The reason why it is such a good model is related to the fact that it shares a common ancestor ("urbilaterian ancestor") with humans that lived 500 million years ago, therefore sharing most of the genes that govern most modern organismal development and disease, such human and nematode. This is extremely important because many genetic and developmental experiments are impossible in humans (technically and ethically). Some evasive procedures associated with the experiment are extremely time-consuming or cost-intensive. *C. Elegans* presents itself as a reliable alternative because of its short generation time (four days), and due to its complete anatomy also

being known. The adult hermaphrodite has exactly 959 cells, while the adult male has exactly 1031 cells and both are transparent. This allows researchers to relate behaviors to particular cells, to trace the effects of genetic mutations and gain relevant insights into the mechanisms of development and ageing. Therefore, due to evolutionary conservation of gene function, *C. Elegans* is the ideal model organism to trace basic genetic mechanisms of human development and disease such as cancer and neurodegenerative diseases. *C. Elegans* was the first multicellular eukaryotic organism having its whole genome sequenced.

Escherichia Coli (Bacteria)

The *Escherichia Coli* (*E. Coli*) can be found in the intestine of warm blood organisms. The reasons why this bacterium is such a good model are firstly because it is a simple organism and secondly due to the fact that it can be cultured and grow easily and inexpensively in laboratory. However, the main reason why its heavily tested and trialed even being a bacterium, is related to the fact that its basic biochemical mechanisms are common to the human organism. Another important aspect is that *E. Coli* was the reason for first understanding the transcription factors that activate and deactivate genes in the presence of a virus. From that point on, *E. Coli* was used as a host in genetic engineering and especially in health, producing several types of proteins, encoding them with the majority of human genes that are applied as medicinal drugs. There are several variants of *E. Coli*, most of them harmless. However, some of them can be lethal being responsible for product recall due to food contamination or food poisoning. This bacterium was the first prokaryotic model organism to have his genome sequenced (K12 strain), having a single circular chromosome with 4.6 million base pairs.

Saccharomyces cerevisiae (Yeast)

Saccharomyces cerevisiae is recognized as the key factor in brewing for centuries. The main reasons for it being considered a model organism are related to its easy culture, fast growing process and inexpensive production in laboratory. Being eukaryotes, they share the same complex internal cell structure of plants or animals without the high percentage of junk DNA present in more complex eukaryotes facilitating research. Since *S. cerevisiae* is biochemically very similar to the human organism, many studies about the molecular processes involved in cell cycle, meiosis, recombination, DNA reparation, ageing and other fundamental areas of biology were possible. *S. cerevisiae* was the first eukaryotic genome to be completely sequenced being composed of 12 chromosomes containing approximately 12,2 million base pairs.

Mus musculus (Mouse)

The *Mus Musculus* (Mouse) is the most famous model organism because is the mostly used mammal in medicine and biology scientific communities. The main reasons why is such a good model are related with being a mammal and therefore it has organs and development processes that are very similar to the human organism; then they are easily reproducible, grow fast, and very easy to maintain and manipulate in laboratory; finally mice suffer from the most diseases and calamities that affect mankind where mice have an extremely important role in the development of new pharmaceuticals for humans. The Mouse genome consists of 40 chromosomes with 2,63 billions of amino acids.

As described before we had made analysis for the four domains of life where the conclusions were: Dark Proteome is mostly not disordered, mostly not compositionally biased, mostly not transmembrane, but more important and unexpectedly, it's mostly "Unknowns Unknowns" (5). Dark Proteins portion of "Unknowns Unknowns" in Eukaryota is more than 50% being composed of ordered, globular and with low compositional bias proteins. In the case of Bacteria this percentage is over 50% and in case of Archaea reached almost the 70%. Finally, in Viruses this percentage reached almost 75% (5). There were several questions raised at that time, that are still valid today, such as: Could we detail more dark proteins location and environment? Could we detail even more its functions? And by life domain? What about organisms?

2. Materials and Methods

Dataset: The set of protein sequences selected for this work were convenient from Swiss-Prot release of July 2016 (6). The protein structures were extracted from PDB on July 2016. Predictions from PSSM2 (4), PMP (8) and Predict Protein (9) are versions from July 2016. Finally, Protein-Protein Interaction (PPI) information is convenient from STRING (10) also from July 2016. The Swiss-Prot dataset is composed of 550,116 proteins and divided in four kingdoms: 19,370 protein sequences from Archaea, 332,327 from Bacteria, 181,814 from Eukaryota and 16,605 from Viruses. The number of proteins sequences for each model organism are: 14,349 protein sequences for Arabidopsis, 3,652 for C Elegans, 669 for E. Coli, 42 for S. cerevisiae, 16,747 for Mouse and finally 20,209 for Human.

Mapping Darkness: For each Swiss-Prot protein, each residue was categorized as “non-dark” if it met either one of the following criteria: if the residue was aligned onto the “ATOM” record of any PDB entry (1) in the corresponding Aquaria matching structures entry (criterion A); or if the residue was aligned onto a PDB entry in the corresponding UniProt entry (criterion B). All other residues were categorized as “dark.” We then calculated a “darkness” score (D) as defined in (5). If $D=0$ means it is PDB or white protein, otherwise if $D=1$ means it is a dark protein. If $0 < D < 1$ means it is a grey protein with grey regions containing dark regions (5).

Dark and non-Dark Percentages: The percentages displayed for “dark” proteins, “dark” regions, grey regions and PDB regions present in the above sets (domains of life and model organisms) consist first in obtaining both “dark” and PDB proteins in the sets mentioned above. Next, “dark” as well as, “non-dark” regions are mapped, subtracting the “dark” proteins from the former, and subtracting the PDB proteins from the later, obtaining the cardinality of “dark” regions and “non-dark” regions. If we divide the above cardinalities by the total amount of dark and non-dark regions, we obtain the percentages presented in Figures 1 and 2.

Annotation Enrichment: The functional analysis by comparing annotations between dark and non-dark proteins in a reliable manner, by application of annotation enrichment for the ‘Description’ (DE) field, where now extended for the ‘Features’ (FT) field of the Swiss-Prot proteins through Fisher exact tests (11,12) followed by the Benjamini-Hochberg false discovery correction (13,6) with α , the fraction of false positives considered acceptable, set to 1%, and accepting only annotations with an adjusted p value of $\leq 1\%$, calculated via:

$$p^{adjusted} = \text{Min}[p \times n / (k + 1), 1]$$

where p is from Fisher’s test, n is the total of number of annotations in the set, and k is the rank of the largest p-value that satisfies the false discovery criteria as in (6). This approach was then repeatedly applied to compare dark and non-dark proteins across various sets of organisms.

Tree Maps. From the ‘Description’ enrichment analysis results, we selected 21 (of 25) subcategories judged to be most significant and visualized them using a tree map (14). For the ‘Features’ enrichment analysis results we have selected 36 (of 39) subcategories. The removed subcategories included those with relatively few results—or results with relatively high adjusted P values—as well as subcategories such as “Similarity,” which only give information about groups of very similar proteins and the specific functions they perform; although interesting, these specific annotations do not reveal more general properties of dark proteins. In Figs. 3,4,5,6,7,8 and 9 the results were displayed using the D3 zoomable tree map library (bost.ocks.org/mike/treemap); some annotation terms have also been reworded to improve readability.

Mapping Autonomy per protein. For every human protein we evaluated its autonomy, i.e., using STRING (10) we had counted with how many others it interacts with. The STRING scheme classifies its functional link confidence into three different scores (15): low (<400), medium ($400 < \text{score} < 700$) and

high (>700) confidence scores measuring the confidence in pair-wise functional interactions of the networks produced. Even assuming that sequence data is accurate, computational tools can introduce noise when generation sequence similarity data occurs. Taking this noise into account, it is suggested to set a cut-off score above which an interaction is highly probable. In terms of functional classification accuracy what matters is high confidence score 700 or higher (16), however low and medium confidence were done for comparison purposes (results not shown). Therefore, for each Swiss-Prot protein, we categorized its autonomy as:

$$\text{Autonomy Score} = \begin{cases} 1 - 0. N & \text{if } m(N) = 0 \text{ and } 0 \leq N \leq 900 \\ 0 & \text{if } m(N) \neq 0 \text{ and } N > 900 \end{cases}$$

where $m(N)$ indicates the number of matches that occur for link score of N . This means if a protein has $m(0)$ equals to zero matches, then the protein is fully autonomous because at the lowest quality cut-off score no interactions occur between it and other proteins. On the other hand, if at the highest cut-off score still exists interactions with other proteins (i.e., $m(900)$ is not zero) then it can be concluded that the protein is completely non-autonomous.

Dark Genes. For each chromosome in Homo Sapiens, we then constructed a list of dark proteins sorted by the position of the central nucleotide of the corresponding gene, determined using UCSC assembly hg19 (17). In some cases, due to gene duplication, multiple copies of the same dark protein are annotated as arising from multiple genes in the same chromosome; in such cases, we considered only the first occurrence, and removed all other copies from the list. For each chromosome, we then calculated the longest run of dark proteins, and assigned a p value by calculating how many times a run with the same number of dark proteins or more occurred by chance in 1,000 random re-orderings of proteins along the chromosome. Note that the cluster results are very conservative where the chance of a false positive is 1/1000 on a per-chromosome basis; thus, there are probably more such 'dark' gene clusters.

Dark Tissues. Finally, we have used ProteomicsDB (18) that contains mass-spectrometry data from protein expression measurements from 16,857 liquid chromatography tandem-mass-spectrometry (LC-MS/MS) experiments involving human tissues, cell lines, body fluids including data from PTM studies and affinity purifications. To obtain the normalized intensity values for each protein from ProteomicsDB, the proteinexpression API was used. These values measure the relative abundance of peptides of each protein in a specific sample in a logarithmic scale, where we didn't find any mass-spectrometry data for 1,391 dark proteins and 2,762 non-dark proteins, considering these empty entries as 0.

3. Results

3.1. Dark Proteome Database Status

This work tries to answer the questions formulated at the introduction, starting by presenting the status of DPD (6) in July 2016 (Fig. 1), i.e., the percentage of dark proteins, dark regions, grey regions and PDB regions as defined in (5) in the four domains of life plus the six model organisms described above. Using the more stringent definition of Darkness as defined in (5) we can observe the status of DPD (Fig. 2) including the PMP predictions for the same four domains of life plus the six model organisms.

Figure 1. Darkness Map per Life Domain and per Model Organism

Figure 2. Darkness Map per Life Domain and per Model Organism with PMP

Comparing the actual version of DPD with PMP (Fig. 1) and without PMP (Fig. 2), we can observe marginal differences either in the domains of life or in the model organisms studied in this work. Therefore, henceforth this study will be only focused in DPD version without PMP.

Comparing now the initial version of DPD (5) (Fig. S1) with the current version of DPD (6) and starting by the domains of life we can observe that dark proteins (from: E:15.2%, B:5.3%, A:5.8% V:28.1% to: E:14.3%, B:4.9%, A:5.5, V:24.3%) and dark regions (from: E:28.8%, B:8.2%, A:7.9%, V:26.3% to: E:28.9%, B:7.7%, A:7.8, V:24.9%) percentages had decreased compared with the previous version of DPD (5), while grey proteins (from: E:52.1%, B:84.6%, A:81.8%, V:41.7% to: E:52.6%, B:85.4%, A:81.9, V:45.4%) and white (PDB) regions (from: E:3.8%, B:1.8%, A:4.5%, V:3.9% to: E:4.2%, B:2.0%, A:4.8, V:4.3%) the percentages had increased (Fig. 1).

Focusing now on model organisms and performing exactly the same reasoning, as the one above, we observe the same tendency (Fig S1), i.e., we can observe that dark proteins (from: Ar:13.6%,

CE:17.2%, EC:17.8%, SC:12.7%, MM:15.4%, HS:16.7% to: Ar:14.0%, CE:15.9%, EC:18.2%, SC:10.9%, MM:15.0%, HS:15.9%) and dark regions (from: Ar:26.8%, CE:29.4%, EC:15.2%, SC:30.3%, MM:35.3%, HS:35.5% to: Ar:27.2%, CE:29.9%, EC:14.3%, SC:29.2%, MM:35.2%, HS:35.8%) percentages had decreased in general (except for Arabidopsis, E. Coli and Yeast) compared with the previous version of DPD (5), while grey proteins (from: Ar:57.9%, CE:51.0%, EC:54.4%, SC:53.8%, MM:46.2%, HS:36.2% to: Ar:57.2%, CE:52.6%, EC:54.1%, SC:56.8%, MM:46.4%, HS:35.2%) and white (PDB) regions (from: Ar:1.7%, CE:1.4%, EC:12.7%, SC:3.2%, MM:3.1%, HS:11.7% to: Ar:1.7%, CE:1.6%, EC:13.3%, SC:3.1%, MM:3.4%, HS:13.1%) the percentages had increased in general (Fig. 1).

It can be concluded by looking at the previous results that the general knowledge concerning the four domains of life has increased since the number of dark regions and dark proteins percentages decrease while the grey and white regions percentages increased. In model organisms the previous conclusion could also apply but not so straightly because there were dark regions and dark proteins areas that expanded while grey and white areas that shrunk even if marginally.

However, if we look at the overall picture (Fig. 1), even with this increase in PDB and grey regions, we conclude that for four domains of life the percentage of the dark proteome is still very high in Eukaryotes (43.0%) and in Viruses (49.2%) and very low in Archaea (13.3%) and in Bacteria (12.6%). Considering PMP, the scenario doesn't get better with 38.6% of darkness present in Eukaryotes, 47.7% in Viruses, 10.6% in Archaea and 10.8% in Bacteria (Fig. 2).

Looking at the model organisms the view is not much different with Ar:41.2%, CE:45.8% EC:32.5%, SC:40.1%, MM:50.2%, HS:51.7%, i.e., with around half of their proteome still in the dark (Fig 1). Considering PMP we obtain Ar:35.5%, CE:36.9% EC:31.1%, SC:38.8%, MM:41.8%, HS:42.8%, i.e., more than one third of each organism (except E. Coli) remains in the dark (Fig. 2).

3.1.1. Domains of Life

Going deeper we wanted to analyze and visualize these dark proteins by domain of life, and to do so we had used TreeMaps to analyze Swiss-Prot fields 'Description' (DE) (Fig. 3) and 'Features' (FT) (Fig. 4) using "Annotation Enrichment" (See Methods) to point towards reliable conclusions.

Observing Swiss-Prot proteins through the TreeMap (with 21 functional categories) of 'Descriptions' field (DE) by life domain show the following:

Archaea dark proteins (Fig. 3A) in "Subcellular Location" are over-represented in 'Cell membrane', 'Chloroplast inner membrane', 'Cell inner membrane' acting as 'Lipid anchors' and being 'Secreted'. Dark proteins are under-represented in 'Cytoplasm'. Dark proteins in this domain of life are over-represented in "Tissue" like 'Venom Gland' and 'Venom Gut' as well as, in 'Skin (including Dorsal) Glands' and 'Testis', being under-represented in only three "Tissues": 'Red blood cells', 'Ubiquitous', and 'Widely expressed'. Dark proteins were under-represented in many "Catalytic site" and "Pathway" annotations, where inference often requires similarity to a PDB structure. Dark proteins in Archaea organisms have several "Functions" that are 'Responsible for cell division', 'Proton extrusion', 'Transport of potassium', as well as playing a 'Role in virus cell tropism' among others.

Figure 3. TreeMap showing all annotations (descriptions) over- and under-represented in dark proteins for all the proteins in Swiss-Prot divided by the four domains (details in Table 1 and dataset S1). Functional annotations over- or under-represented in dark proteins. Pooling annotations for all proteins, we used enrichment analysis to find biological functions associated with dark proteins. The tree map shows all over- and under-represented annotations (dark and blue, respectively) in 21 functional categories; cell area indicates annotation significance (scaled to $-\log_{10}(P)$, using the adjusted P value from Fisher's exact test – see methods).

Table 1 Annotations enriched (Features field - FT) in dark proteins from Eukaryota (only the first 20 entries).

Non-dark	Dark	Ratio	Total	Fisher's p value	Adjusted p value	Annotation sub-category	Annotation
1	380	2397.28	381	0	0	SIMILARITY	Belongs to the Casparian strip membrane proteins (CASP) family. {ECO:0000305}.
1722	1900	6.96	3622	0	0	SUBCELLULAR_LOCATION	Membrane {ECO:0000305}; Multi-pass membrane protein {ECO:0000305}.
182	744	25.79	926	0	0	TISSUE_SPECIFICITY	Expressed by the venom duct.
1	376	2372.04	377	2.96E-323	2.31E-318	SUBUNIT	Homodimer and heterodimers. {ECO:0000250}.
966	900	5.878	1866	2.94E-281	1.84E-276	SUBCELLULAR_LOCATION	Membrane {ECO:0000305}; Single-pass membrane protein {ECO:0000305}.
14	276	124.37	290	9.18E-217	4.77E-212	DOMAIN	The presence of a 'disulfide through disulfide knot' structurally defines this protein as a knottin. {ECO:0000250}.
448	557	7.84	1005	1.80E-212	8.02E-208	SUBCELLULAR_LOCATION	Cell membrane {ECO:0000250}; Multi-pass membrane protein {ECO:0000250}.
39	250	40.44	289	9.16E-171	3.57E-166	TISSUE_SPECIFICITY	Testis.
0	196	0	196	4.22E-170	1.46E-165	SIMILARITY	Belongs to the PsbN family. {ECO:0000255 HAMAP-Rule:MF_00293}.
0	194	0	194	2.26E-168	7.06E-164	FUNCTION	May play a role in photosystem I and II biogenesis. {ECO:0000255 HAMAP-Rule:MF_00293}.
0	189	0	189	4.75E-164	1.35E-159	SUBCELLULAR_LOCATION	Plastid, chloroplast thylakoid membrane {ECO:0000255 HAMAP-Rule:MF_00293}; Single-pass membrane protein {ECO:0000255 HAMAP-Rule:MF_00293}.
412	456	6.98	868	6.67E-162	1.73E-157	SUBCELLULAR_LOCATION	Endoplasmic reticulum membrane {ECO:0000250}; Multi-pass membrane protein {ECO:0000250}.
0	184	0	184	9.98E-160	2.39E-155	CAUTION	Originally thought to be a component of PSII; based on experiments in Synechocystis, N.tabacum and barley, and its absence from PSII in T.elongatus and T.vulcanus, this is probably not true. {ECO:0000255
26	217	52.65	243	4.35E-155	9.69E-151	SIMILARITY	Belongs to the conotoxin O1 superfamily. {ECO:0000305}.
87	258	18.71	345	6.35E-146	1.32E-141	SIMILARITY	Belongs to the DEFL family. {ECO:0000305}.
1	155	977.84	156	1.57E-132	3.06E-128	SIMILARITY	Belongs to the protamine P1 family. {ECO:0000305}.
0	145	0	145	5.12E-126	9.40E-122	FUNCTION	Mitochondrial membrane ATP synthase (F1)F(0) ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain. F-type ATPases consist of two structural domains, F(1) - containing the extramembraneous catalytic core and F(0) - containing the membrane proton channel, linked together by a central stalk and a peripheral stalk. During catalysis, ATP synthesis in the catalytic domain of F(1) is coupled via a rotary mechanism of the central stalk subunits to proton translocation. Part of the complex F(0) domain. Minor subunit located with subunit a in the membrane (By similarity). {ECO:0000250}.
0	143	0	143	2.74E-124	4.75E-120	SUBCELLULAR_LOCATION	Mitochondrion membrane; Single-pass membrane protein.
0	142	0	142	2.01E-123	3.29E-119	SIMILARITY	Belongs to the ATPase protein 8 family. {ECO:0000305}.
2313	18	0.05	2331	3.33E-119	5.20E-115	CATALYTIC_ACTIVITY	ATP + a protein = ADP + a phosphoprotein.

Bacteria dark proteins (Fig. 3B) like Archaea are over-represented in "Subcellular Location" like 'Cell membrane', 'Chloroplast inner membrane', 'Cell inner membrane', 'Lipid anchors' and being 'Secreted'. Dark proteins are also under-represented in 'Cytoplasm'. Dark proteins were under-represented in many "Catalytic site", "Pathway" and "Subunit" (namely 'Ribosomal') annotations. Dark proteins in Bacteria organisms have several "Functions" that are 'Responsible for cell division', 'Transport of potassium', as well as, being 'Catalyzers' and being involved in 'Initiation control of chromosome replication' among others.

Eukaryota dark proteins (Fig. 3C) are over-represented in "Subcellular Location" like many specific secretory tissues and exterior environment like 'Venom Gland', 'Venom Gut', in 'Skin (including Dorsal) Glands', 'Testis' and 'Milk' and under-represented in the same three "Tissues" annotations like Archaea. Dark proteins were also over-represented in 'Cysteine-rich' domains and 'Disulfide bonds'. Additionally, eukaryotic dark proteins were over-represented in 'Cleavage' and other post-translational modifications known to prepare proteins for harsh environments. Dark proteins like Archaea were under-represented in many "Catalytic site" and "Pathway" annotations.

Finally, Viruses dark proteins (Fig. 3D) are over-represented in "Subcellular Location" like 'Virion', 'Virion membrane', 'Host nucleus', 'Host cytoplasm' and 'Host mitochondrion'. Dark Proteins are associated with "Functions" of 'Viral infection', 'Virus transportation', as well as, 'Replication' on 'Hosts'. Dark proteins were under-represented in many annotations like "Catalytic site", "Pathway" and "Enzyme regulation".

We wanted to detail even more the Dark Proteome therefore, we used the 'Features' field (FT) which is a subsection of the 'Description' field (DE) by life domain through TreeMap (with 36 functional categories). Observing Figure 4, allow us to conclude the following:

Archaea dark proteins (Fig. 4A) are over-represented in "Transmembrane" as 'Helical' and in "Topological Domains" as 'Extracellular' and 'Cytoplasmic'. They are also over-represented in "Carbohydr" or "Glycosylation", "Compositional bias" of 'Poly-Glu' and in "Non-Standard" amino acids (Selenocysteine and Pyrrolysine). Dark proteins are under-represented in "Active Sites", "Helixes", "Metal", "NP-bind" and "Binding".

Bacteria dark proteins (Fig. 4B) like Archaea are over-represented in "Transmembrane" as 'Helical' and in "Topological Domains" as 'Cytoplasmic' and 'Periplasmic'. They are also over-represented in "Lipid" or "Crosslink", "Compositional bias". Dark proteins are under-represented in "Metal", "Binding", "Active Sites", "Domain", "Regions" and "NP-bind".

Eukaryota dark proteins (Fig. 4C) are over-represented in "Topological Domains" as 'Extracellular', 'Cytoplasmic' and 'Lumenal'. Dark proteins were also over-represented in "Compositional bias", "Lipid", "Motifs" and "Unsure". Dark proteins are under-represented in many "Binding", "Metal", "NP-bind", "CA-bind" and "Crosslink" annotations.

Finally, in Viruses dark proteins (Fig. 4D) are over-represented in "Transmembrane" and in "Topological Domains" as 'Intravirion' and 'Virion Surface'. Dark Proteins are also over-represented in "Compositional bias", "Mutagene" and "Cleaved Initiator Methionine". Dark proteins were under-represented in many annotations like "Binding", "Active Sites", "Metal", "Sites" and "Regions".

Figure 4. TreeMap showing all annotations (features) over- and under-represented in dark proteins for all the proteins in Swiss-Prot by the four domains of life and divided into 39 functional categories (details in Table 2 and dataset S2).

Table 2: Annotations enriched (Descriptions field – DE) in dark proteins from Eukaryota (only the first 20 entries).

Non-dark	Dark	Ratio	Total	Fisher's p value	Adjusted p value	Annotation sub-category	Annotation
96211	24496	3.25	120707	0	0	transmem	Helical; (Potential)
15723	3753	3.05	19476	0	0	signal	Potential
45	562	159.48	607	0	0	mod_res	Phenylalanine amide
4152	1677	5.16	5829	0	0	topo_dom	Lumenal (Potential)
69	524	96.98	593	0	0	mod_res	Leucine amide
35139	6509	2.37	41648	0	0	topo_dom	Cytoplasmic (Potential)
16045	11	0.01	16056	0	0	binding	Substrate (By similarity)
6648	3000	5.76	9648	0	0	non_ter	
10317	2823	3.49	13140	0	0	coiled	Potential
110128	0	0	110128	0	0	strand	
27108	0	0	27108	0	0	turn	
103913	0	0	103913	0	0	helix	
44	312	90.55	356	5.19E-301	1.79E-296	mod_res	Valine amide
8891	1	0	8892	1.69E-289	5.43E-285	np_bind	ATP (By similarity)
24535	3794	1.97	28329	2.95E-287	8.82E-283	mod_res	Phosphoserine (By similarity)
904	596	8.42	1500	1.95E-273	5.46E-269	unsure	L or I
8122	2	0	8124	8.37E-262	2.21E-257	act_site	Proton acceptor (By similarity)
1186	641	6.90	1827	1.49E-257	3.70E-253	non_cons	
401	414	13.18	815	8.24E-242	1.94E-237	mod_res	Pyrrolidone carboxylic acid
6967	0	0	6967	5.48E-229	1.23E-224	np_bind	GTP (By similarity)

3.1.2. Model Organisms

Arabidopsis has 41.2% of its proteome in the dark (Fig. 1). The conclusions that we can infer through the analysis of the corresponding TreeMap (Fig. 5A) for ‘Descriptions’ (DE field of Swiss-Prot files) are: that dark proteins are over-represented in “Subcellular Location” like ‘Endoplasmic reticulum membrane’ either in dark proteins with ‘Single-pass’ or ‘Multi-pass’. These dark proteins are also ‘Secreted’ in the ‘Extracellular space’ or through the ‘Cell wall’. Dark proteins most evident “Functions” are related with ‘Transcription factors’ as well as, with ‘Regulation of cell fate’, and even with ‘Regulation of the plant stress, growth and development’. Dark proteins are under-represented in ‘Chloroplast’ and in ‘Cytoplasm’. Analyzing the results of the corresponding TreeMap (Fig. 5B) for ‘Features’ (FT field of Swiss-Prot files) we can observe that: the dark proteins are located mostly in the extension of “Transmembrane” regions where its “Topological domain” are ‘Cytoplasmic’ and ‘Extracellular’. Dark proteins are common in “Signal” sequences (prepeptides) and related with transcription factors being also associated with “Disulfides”. They are under-represented in “Helix”, “Binding”, “Metal” and “NP-bind” (Fig. 10A).

The *C. Elegans* organism contains 45.8% of dark proteome (Fig. 1). Analyzing again the TreeMap of 'Descriptions' (Fig. 6A) we conclude that dark proteins are mostly located in the 'Cell membrane' specially in dark proteins with 'Multi-pass' or 'Single-pass'. The main "Functions" of the dark proteins are being "Structural on the gap junctions", being also present in "Neuropeptides". Turning now our attention to the TreeMap (Fig. 6B) for 'Features' we can deduce that dark proteins are located essentially in the extension of 'Transmembrane' regions ('Helical'), and like in *Arabidopsis*, they are also 'Signal' sequences. They are under-represented in "Disulfides", "Helix", "Binding" and "NP-bind" (Fig. 10B).

E. Coli contains 32.5% of dark matter in its proteome. Attending TreeMap (Fig. 7A) of 'Descriptions' of E. Coli dark proteins are over-represented in larger number at the 'Cell outer membrane' mainly as a 'Lipid anchor'. However, they are over-represented also at 'Cell inner membrane' where they are 'Secreted' in a slighter less quantity. These dark proteins also interact with themselves to form ligaments. Functions associated with these dark proteins, not surprisingly include the 'Conjunctive DNA transfer (CDT) which is the unidirectional transfer of ssDNA plasmid from a donor to a recipient cell which is the central mechanism by which antibiotic resistance and virulence factors are propagated in bacterial populations. Dark proteins can also be associated with 'Lysis' proteins. According with 'Features' TreeMap (Fig. 7B) of E. Coli dark proteins are over-represented in "Transmembrane (Helical)" and are associated with "Lipid" bonds. They are under-represented in Helix (Fig. 10C).

Figure 7. (A) TreeMap showing all annotations (descriptions) over-represented in dark proteins for organism E. Coli (details in dataset S1). **(B)** TreeMap showing all annotations (features) over-represented in dark proteins for organism E. Coli (details in dataset S2).

S. Cerevisiae has 40.1% of its proteome on dark side, and there are not sufficient proteins to infer conclusions about it using Fisher-tests for ‘Descriptions’ and ‘Features’.

The Mus Musculus have 51.7% of dark Proteome. Concerning ‘Descriptions’ (Fig. 8A) the dark proteins of the mouse organism are over-represented in ‘Membrane’, ‘Golgi Apparatus membrane’, ‘Mitochondrion’, ‘Endoplasmic Reticulum membrane’ with ‘Multi-pass’ and ‘Single-pass’ proteins. Dark proteins are under-represented in ‘Cell membrane’. They have “Tissue Specificity” in the ‘Lower and middle cortical regions of the hair shaft in both developing and cycling hair’. Dark proteins also ‘Interact with hair keratin’ having the purpose or function of keeping hair strong. In the ‘Hair cortex, hair keratin intermediate filaments are embedded in an interfilamentous matrix, consisting of hair keratin-associated proteins (KRTAP), which are essential for the formation of a rigid and resistant hair shaft through their extensive disulfide bond cross-linking with abundant cysteine residues of hair keratins. The matrix proteins include the high-sulfur and high-glycine-tyrosine keratins’. Focusing on ‘Features’ (Fig. 8B) dark proteins are over-represented in “Transmembrane”,

[illegible]

The last organism studied in this work was the Homo Sapiens (Human) proteome, finding that over half of it (51.7%) was dark (Fig. 1). The results for Human enrichment analysis 'Description' (Fig. 9A) gives dark proteins over-representation at "Subcellular Location" like 'Membrane' ('Multi-pass' and 'Single-pass') were they 'Shuttles between nucleolus and cytoplasm' being also 'Secreted'. About "Tissue Specificity" they are over-represented in 'Testis', 'Testis (tumor tissues)', 'Melanoma' and 'Carcinoma (bladder and lungs)'. Concerning "Functions" can be observed that dark proteins are directly linked with 'Tumorigenesis', 'Tumor antigens' and 'Retroviral replication'. In "Caution" like stated in (5) although using Swiss-Prot partly addresses the possibility that dark proteins may actually be unrecognized long noncoding RNA or may arise from pseudogenes were evidence occurs for a small number of cases. For the human enrichment analysis 'Features' (Fig. 9B), all significant

results are shown in “Transmembrane”, “Coiled”, “Compositional bias” and ‘Cleavage’. Dark proteins are under-represented in ‘Disulfide’ (Fig. 10E). In conclusion we can add for the human case that less was known about the function and subcellular location of dark proteins, 56% shorter ‘CC’ field; missing location data for 56% compared with 22% for non-dark proteins (Fig. 10F).

Figure 9. (A) TreeMap showing all annotations (descriptions) over-represented in dark proteins for organism Human (details in Table 3 and dataset S1). (B) TreeMap showing all annotations (features) over-represented in dark proteins for organism Human (details in Table 4 and dataset S2);

Table 3: Annotations enriched (Description field - DE) in dark proteins from Homo Sapiens (only the first 20 entries).

Non-dark	Dark	Ratio	Total	Fisher's p value	Adjusted p value	Annotation sub-category	Annotation
124	28	18.49	152	9.71E-25	6.65E-20	SUBCELLULAR_LOCATION	Membrane; Single-pass membrane protein (Potential).
0	11	0	11	7.55E-22	2.59E-17	CAUTION	Product of a dubious CDS prediction. May be a non-coding RNA.
162	27	13.64	189	7.11E-21	1.62E-16	CAUTION	Could be the product of a pseudogene.
5	12	196.47	17	5.29E-20	9.05E-16	CAUTION	Product of a dubious CDS prediction.
0	9	0	9	5.27E-18	7.22E-14	MISCELLANEOUS	Encoded by one of the numerous copies of NBPF genes clustered in the p36, p12 and q21 region of the chromosome 1.
0	9	0	9	5.27E-18	6.01E-14	SIMILARITY	Belongs to the beta type-B retroviral envelope protein family. HERV class-II K(HML-2) env subfamily.
0	8	0	8	4.39E-16	4.30E-12	FUNCTION	Retroviral replication requires the nuclear export and translation of unspliced, singly-spliced and multiply-spliced derivatives of the initial genomic transcript. Rec interacts with a highly structured RNA element (RcRE) present in the viral 3'LTR and recruits the cellular nuclear export machinery. This permits export to the cytoplasm of unspliced genomic or incompletely spliced subgenomic viral transcripts (By similarity).
0	8	0	8	4.39E-16	3.76E-12	SUBUNIT	Forms homodimers, homotrimers, and homotetramers via a C-terminal domain. Associates with XPO1 and with ZNF145 (By similarity).
1	8	654.91	9	3.91E-15	2.98E-11	PTM	Specific enzymatic cleavages in vivo yield the mature SU and TM proteins (By similarity).
0	7	0	7	3.66E-14	2.51E-10	SUBCELLULAR_LOCATION	Cytoplasm (By similarity). Nucleus, nucleolus (By similarity). Note=Shuttles between the nucleus and the cytoplasm. When in the nucleus, resides in the nucleolus (By similarity).
3	8	218.30	11	7.02E-14	4.37E-10	SUBUNIT	The surface (SU) and transmembrane (TM) proteins form a heterodimer. SU and TM are attached by noncovalent interactions or by a labile interchain disulfide bond (By similarity).
218	21	7.89	239	2.55E-12	1.46E-08	SUBCELLULAR_LOCATION	Membrane; Multi-pass membrane protein (Potential).
0	5	0	5	2.54E-10	1.34E-06	MISCELLANEOUS	The ancestral BAGE gene was generated by juxtacentromeric reshuffling of the KMT2C/MLL3 gene. The BAGE family was expanded by juxtacentromeric movement and/or acrocentric exchanges. BAGE family is composed of expressed genes that map to the juxtacentromeric regions of chromosomes 13 and 21 and of unexpressed gene fragments that scattered in the juxtacentromeric regions of several chromosomes, including chromosomes 9, 13, 18 and 21.
0	5	0	5	2.54E-10	1.24E-06	SIMILARITY	Belongs to the BAGE family.
0	5	0	5	2.54E-10	1.16E-06	CAUTION	Product of a dubious CDS prediction. Probable non-coding RNA.
215	17	6.47	232	4.87E-09	2.09E-05	SUBCELLULAR_LOCATION	Secreted (Potential).
2	5	204.66	7	5.22E-09	2.10E-05	FUNCTION	Retroviral envelope proteins mediate receptor recognition and membrane fusion during early infection. Endogenous envelope proteins may have kept, lost or modified their original function during evolution. This endogenous envelope protein has lost its original fusogenic properties.
3	5	136.44	8	1.38E-08	5.25E-05	SUBUNIT	Complex I is composed of 45 different subunits.
0	4	0	4	2.11E-08	7.61E-05	CAUTION	No predictable signal peptide.

0	4	0	4	2.11E-08	7.23E-05	SIMILARITY	Belongs to the Speedy/Ringo family.
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Table 4: Annotations enriched (Features field - FT) in dark proteins from Homo Sapiens (only the first 20 entries).

Non-dark	Dark	Ratio	Total	Fisher's p value	Adjusted p value	Annotation sub-category	Annotation
50327	0	0	50327	3.58E-140	5.65E-135	strand	
46207	0	0	46207	6.54E-128	5.15E-123	helix	
7572	217	4.75	7789	2.33E-76	1.22E-71	transmem	Helical; (Potential)
2023	81	6.64	2104	1.29E-38	5.10E-34	coiled	Potential
11972	0	0	11972	3.59E-32	1.13E-27	turn	
12033	5	0.07	12038	4.92E-25	1.29E-20	disulfid	By similarity
0	8	0	8	1.65E-18	3.71E-14	domain	NBPF 3
0	8	0	8	1.65E-18	3.24E-14	domain	NBPF 1
0	8	0	8	1.65E-18	2.88E-14	domain	NBPF 2
152	19	20.73	171	1.84E-18	2.89E-14	compbias	Poly-Arg
3	9	497.59	12	2.13E-18	3.05E-14	motif	Nuclear export signal (Potential)
3	8	442.30	11	2.67E-16	3.51E-12	region	Fusion peptide (Potential)
0	7	0	7	2.75E-16	3.34E-12	domain	NBPF 4
0	7	0	7	2.75E-16	3.10E-12	domain	NBPF 5
1939	46	3.93	1985	2.92E-14	3.07E-10	signal	Potential
0	6	0	6	4.61E-14	4.54E-10	domain	NBPF 6
482	20	6.88	502	6.27E-11	5.81E-07	compbias	Poly-Glu
33	8	40.20	41	1.32E-10	1.16E-06	site	Cleavage (By similarity)
0	4	0	4	1.29E-09	1.07E-05	mutagen	C->S: No significant activity
3356	0	0	3356	3.16E-09	2.49E-05	disulfid	

3.2. Autonomy

Concerning autonomy in Arabidopsis (Fig. 11A) it can be observed that dark proteins have much less interactions in comparison with non-dark proteins which are quite high. Note the small peaks shown near 110 interactions that are a consequence of ribosomal proteins, these peaks will be present in all organisms (except E. Coli) below. The autonomy for C. Elegans follows the same previous pattern of Arabidopsis (Fig. 11B) where dark proteins have much less interactions in comparison with non-dark proteins that are fewer in comparison, but yet quite high. The autonomy for E. Coli has a curious result by comparing it with the previous cases (Fig. 11C), where dark and non-dark proteins have the same number of interactions for high quality (700). The results for Yeast are similar with E. Coli. Concerning Mouse autonomy (Fig. 11D) it can be observed that dark proteins have fewer interactions in comparison with non-dark proteins of Mouse which are quite sound. Autonomy in the Human organism follows the same pattern presented in Mouse (Fig. 11E), where dark proteins have much less interactions in comparison with non-dark proteins of Human however, less than in Mouse organism.

Figure 10. (A) Cellular locations over- and underrepresented of dark proteins in Arabidopsis; (B) Cellular locations over- and underrepresented of dark proteins in C. Elegans; (C) Cellular locations over- and underrepresented of dark proteins in E. Coli; (D) Cellular locations over- and underrepresented of dark proteins in Mouse; (E) Cellular locations over- and underrepresented of dark proteins in Human; (F) Dark proteins in Human have shorter functional descriptions, fewer sequence-specific features, and less complete annotation about subcellular location and tissue distribution.

Figure 11. (A) Protein-proteins interactions (high quality = 700) for Arabidopsis using STRING; (B) Protein-proteins interactions (high quality = 700) for C. Elegans using STRING; (C) Protein-proteins interactions (high quality = 700) for E. Coli using STRING; (D) Protein-proteins interactions (high quality = 700) for Mouse using STRING; (E) Protein-proteins interactions (high quality = 700) for Human using STRING.

3.3. Dark Genes

It was also determined which dark proteins came from sequential genes, finding seven 'dark' gene clusters. Basically, you can take each protein and mapped down to the gene where the protein comes from and mapping down to chromosomes (See Methods), and if we do that proteins from these clusters had many features described above as typical for dark proteins (Table 5).

Table 5: Human gene clusters containing dark proteins.

Gene	Protein	Length	Binds	Bias	Gene	Protein	Length	Binds	Bias
<i>Chromosome 1 (q21.3): P/QK-rich, keratinocyte proteins</i>					<i>Chromosome 17 (q21.2): CS-rich, keratin associated proteins</i>				
LCE5A	Late cornified envelope 5A	118	3	21	KRTAP3-3	Keratin associated protein 3-3	98	19	
CRCT1	Cysteine-rich C-terminal 1	99	2	25	KRTAP3-2	Keratin associated protein 3-2	98	19	
LCE3E	Late cornified envelope 3E	92	2	16	KRTAP3-1	Keratin associated protein 3-1	98	18	
LCE3E	Late cornified envelope 3E	92	2	17	KRTAP3-1	Keratin associated protein 3-1	98	24	
LCE3D	Late cornified envelope 3D	92	2	19	KRTAP1-5	Keratin associated protein 1-5	174	25	
LCE3C	Late cornified envelope 3C	94	4	19	KRTAP1-1	Keratin associated protein 1-1	177	27	
LCE3B	Late cornified envelope 3B	95	2	24	KRTAP2-1	Keratin associated protein 2-1	128	27	
LCE3A	Late cornified envelope 3A	89	1	21	KRTAP2-1	Keratin associated protein 2-1	128	35	
LCE2D	Late cornified envelope 2D	110	1	20	KRTAP4-11	Keratin associated protein 4-11	195	37	
LCE2C	Late cornified envelope 2C	110	21		KRTAP4-12	Keratin associated protein 4-12	201	37	
LCE2B	Late cornified envelope 2B	110	22		KRTAP4-4	Keratin associated protein 4-4	166	36	
LCE2A	Late cornified envelope 2A	106	1	20	KRTAP4-3	Keratin associated protein 4-3	195	35	
LCE4A	Late cornified envelope 4A	99	3	18	KRTAP4-2	Keratin associated protein 4-2	136	34	
KPRP	Keratinocyte proline-rich protein	579	1	20	KRTAP4-1	Keratin associated protein 4-1	146	36	
LCE1F	Late cornified envelope 1F	118	22		KRTAP17-1	Keratin associated protein 17-1	105	19	
LCE1E	Late cornified envelope 1E	118	1	22	<i>Chromosome 21 (q22.11): GYSC-rich, keratin-associated proteins</i>				
LCE1D	Late cornified envelope 1D	114	1	22	CLDN17	Claudin 17	224	1	14
LCE1C	Late cornified envelope 1C	118	1	21	CLDN8	Claudin 8	225	1	10
LCE1B	Late cornified envelope 1B	118	20		KRTAP24-1	Keratin associated protein 24-1	254	2	19
LCE1A	Late cornified envelope 1A	110	1	14	KRTAP25-1	Keratin associated protein 25-1	102	21	
LCE6A	Late cornified envelope 6A	80	17		KRTAP26-1	Keratin associated protein 26-1	210	2	18
SMCP	Sperm mitochondria-associated	116	29		KRTAP27-1	Keratin associated protein 27-1	207	2	16
SPRR4	Small proline-rich protein 4	79	22		KRTAP23-1	Keratin associated protein 23-1	65	2	20
SPRR3	Small proline-rich protein 3	169	29		KRTAP13-2	Keratin associated protein 13-6, pseudogene	175	23	
SPRR1B	Small proline-rich protein 1B	89	38		KRTAP13-1	Keratin associated protein 13-1	172	23	
SPRR2D	Small proline-rich protein 2D	72	38		KRTAP13-3	Keratin associated protein 13-3	172	22	
SPRR2A	Small proline-rich protein 2A	72	1	39	KRTAP13-4	Keratin associated protein 13-4	160	21	
SPRR2B	Small proline-rich protein 2B	72	1	39	KRTAP19-1	Keratin associated protein 19-1	90	42	
SPRR2E	Small proline-rich protein 2E	72	36		KRTAP19-2	Keratin associated protein 19-2	52	27	
SPRR2F	Small proline-rich protein 2F	72	40		KRTAP19-3	Keratin associated protein 19-3	81	43	
SPRR2G	Small proline-rich protein 2G	73	26		KRTAP19-4	Keratin associated protein 19-4	84	27	
LELP1	Late cornified envelope-like	98	21		KRTAP19-5	Keratin associated protein 19-5	72	39	
<i>Chromosome 4 (q13.3): P-rich, mouth and digestive secreted proteins</i>					KRTAP19-7	Keratin associated protein 19-7	63	2	33
CSN1S1	Casein alpha s1	185	2	11	KRTAP6-2	Keratin associated protein 6-2	62	32	
CSN2	Casein beta	226	2	17	KRTAP6-1	Keratin associated protein 6-1	71	38	
STATH	Statherin	62	2	11	KRTAP20-1	Keratin associated protein 20-1	56	36	
HTN3	Histatin 3	51	1	14	KRTAP20-2	Keratin associated protein 20-2	65	37	
HTN1	Histatin 1	57	2	12	KRTAP20-3	Keratin associated protein 20-3	44	4	25
C4orf40	Proline-rich protein 27	219	21		KRTAP21-1	Keratin associated protein 21-1	79	2	35
ODAM	Odontogenic, ameloblast associated	279	15		KRTAP8-1	Keratin associated protein 8-1	63	24	
C4orf7	Follicular dendritic cell secreted	85	19		KRTAP11-1	Keratin associated protein 11-1	163	15	
CSN3	Casein kappa	182	2	16	KRTAP19-8	Keratin associated protein 19-8	63	4	35
SMR3B	Salivary gland androgen regulated	79	1	39	<i>Chromosome X (p11.23): EPG-rich, GAGE and PAGE family proteins</i>				
MUC7	Mucin 7, secreted	377	4	20	GAGE10	G antigen 10	116	17	
AMTN	Amelotin	209	1	15	GAGE12J	G antigen 12J	117	16	
AMBN	Enamel matrix protein	447	1	15	GAGE12F	G antigen 6	117	17	
IGJ	Immunoglobulin J chain	159	1	9	GAGE13	G antigen 13	117	17	
UTP3	Processome component	479	1	13	GAGE2E	G antigen 8	116	17	
<i>Chromosome 11 (q12.1-q12.2): LS-rich, transmembrane complex members</i>					GAGE2D	G antigen 8	116	16	
MS4A3	Member 3	214	13		GAGE2C	G antigen 2C	116	18	
MS4A2	Member 2, receptor for	244	12		GAGE12B	G antigen 12B	117	17	
MS4A6A	Member 6A	248	2	14	GAGE2A	G antigen 2A	116	17	
MS4A4E	Putative member 4E	132	2	11	GAGE1	G antigen 6	139	14	
MS4A4A	Member 4	239	1	11	GAGE4	Cancer/testis antigen 4.4	117	17	
MS4A6E	Member 6E	147	2	16	PAGE1	P antigen family, member 1	146	18	
MS4A7	Member 7	240	1	15	PAGE4	P antigen family, member 4	102	15	
MS4A5	Member 5	200	13		<i>Chromosome X (p11.22): EP-rich, contains XAGE family proteins</i>				
					XAGE2B	X antigen family, member 2B	111	13	
					XAGE1B	G antigen member; Cancer/testis antigen 12.1	81	15	
					SSX7	Synovial sarcoma, X breakpoint 7	188	12	
					SSX2B	Synovial sarcoma, X breakpoint 2B	188	12	
					SPANXN5	SPANX family, member N5	72	14	
					XAGE5	X antigen family, member 5	108	12	
					XAGE3	X antigen family, member 3	111	15	
					FAM156A	Family with sequence similarity 156, member B	213	12	

Length indicates the number of amino acids; ‘Binds’ indicates the number of known binding partners in the same cluster from STRING (10); ‘Bias’ indicates the largest single amino acid composition (e.g., a value of ‘42%’ indicates that one amino acid accounts for 42% of the entire sequence) – the most frequently occurring amino acids are given for each cluster (e.g., ‘CS-rich’ indicates Cys is the most common, followed by Ser). The proteins arising from these gene clusters exhibit typical characteristics of dark proteins: they tend to be short, have few known interactions, have atypical amino acid composition, and are often secreted, transmembrane, or skin-associated. The 1q21.3 cluster arises from gene duplication (22); it contains many skin proteins with significant compositional bias. The 4q13.3 cluster does not appear to have been previously characterized; it contains proteins related to the mouth, salivary glands, and secretion, implying that these genes share related functions. The 11q12 cluster arises from gene duplication during vertebrate evolution (18); it contains proteins that all have a 4-pass membrane-spanning region and are components of a multimeric receptor complexes. The 17q21.2 and 21q22.11 clusters have also been previously identified (19,20); they contain hair-associated proteins. The Xp11.23 and Xp11.22 clusters are both very recent evolutionary developments (21); they contain proteins that are expressed only in testis and in cancer - some are also unique to human

3.4. Dark Organs

Finally, using ProteomicsDB (18) we looked at all the proteins that were expressed in 69 tissues, where every tissue has a list of expressed proteins and a level of abundance. What we have done was inspecting each tissue (like for instance, the brain), and observe which proteins were highly expressed and which fraction of those proteins were dark, associated with a darkness value, not for each protein, but for each tissue. The tissue that has the highest level of darkness is the heart, which is very interesting, since it's the tissue that is associated with heart disease, one of the main cause of death in humans (Table 6).

Table 6. Tissues with the highest levels of darkness (only the first 25 entries)

Rank	Tissue	Ratio Dark Residues
1	Heart	50%
2	Cervical Mucosa	50%
3	Natural Killer Cell	50%
4	Lung	49%
5	Testis	49%
6	Rectum	49%
7	Proximal Fluid Coronary Sinus	49%
8	Pancreas	49%
9	B. Lymphocyte	49%
10	Colon Muscle	49%
11	Bone Marrow Stromal Cell	48%
12	Hair Follicle	48%
13	Cytotoxic T Lymphocyte	48%
14	Helper T Lymphocyte	48%
15	Colon	48%
16	Ovary	48%
17	Stomach	48%
18	Spinal Cord	47%
19	Placenta	47%
20	Vitreous Humor	47%
21	Blood Platelet	47%
22	Prostate Gland	47%
23	Retina	47%
24	Salivary Gland	47%
25	Uterus	46%

4. Discussion

Our previous work (5) didn't point out solutions, but it opened a new field to be explored. The focus of this study however, was the Homo Sapiens and other model organisms where it can be concluded that the amount of dark proteome present in them is still high, whereas in higher eukaryotes like mouse and human, it is around 50%. The results presented above are consistent with previous works (6, 23), since Arabidopsis dark proteins are mainly located in extracellular space, cellular membrane and endoplasmic reticulum membranes. C. Elegans dark proteins are present again in cell membrane where they are secreted. In the E. Coli case, it is reconfirmed that they are present in inner and outer membrane. Finally, in higher eukaryotic organisms like mouse, we observed that dark proteins are located in endoplasmic reticulum and in mitochondrion membrane, which is consistent with the previous results that state that dark proteins are mostly over-represented in specific secretory tissues and exterior environments, being also related to cancer endogenous retroviral proteins in the human organism (6). Therefore, it was shown that dark proteins are not

uniformly distributed throughout the different areas of the cell in organisms, where their presence is more common in some regions than in others. There are a lot of them in membranes, cell membranes or associated with transmembrane regions and cleavage, but they are less common in cytoplasm, where many globular proteins perform their activity.

Concerning functions, the results confirmed that dark proteins perform a wide spectrum of functions depending on the organism in question, being more focused in simpler organisms and wider in higher organisms (6). Again, it was found that a vast amount of them is programmed to live outside the cell, where many are associated with secretion (through secretory glands and guts) or with extracellular areas in tissues, being an indicator that they possibly are designed for being defensive agents against external threats such as bacteria and/or virus. But we also observed that some of these dark proteins are subject to post-translational modifications, therefore being chemically modified after translation be applied.

Concerning Autonomy, up to now there is no comprehensive map of all relevant functionally for PPI's in simple or complex organisms. The existence of this map is of crucial importance to understand cellular behavior. Several databases started to flourish helping in the construction of this global protein interactions map. Some databases are dedicated to register interaction experiments such as physical binding detection among proteins (24, 25, 26, 27); others are centered on specific model organisms (28, 29). However, there are two difficulties: the first is the "tsunami" of genome and proteome sequencing information that must be processed putting the above map in standby; The second difficulty is in the way proteins interact i.e., they also interact through indirect associations such as shared pathways which are not registered in interaction databases, but instead are registered in pathway databases (30, 31). This is our contribution to the above map, especially to its dark side. The results show clear evidence that - independently of the organism evaluated - dark proteins have significantly fewer interactions with other proteins, in comparison with non-dark proteins. In general, we can conclude that dark proteins are more independent and autonomous than non-dark proteins. Therefore, the DPD is a map for the dark proteome at the present time where the model organisms described are already available together with its functional analysis, augmenting the knowledge about them, where we have work in progress for all the remaining organisms.

5. Conclusions

Five hundred years ago, very little of the Earth was known. People suspect that it was a sphere, with land and water and they had roughly mapped out Europe, but that was it. Knowing what they didn't know gave the Portuguese explorers like Vasco da Gama, Pedro Álvares Cabral and Fernão de Magalhães a direction in which to head — the same principle applies to science and discovery today. We have been able to identify regions within each protein that are different to any region where the structure has been determined experimentally. This unknown area is called the 'dark proteome' and actually accounts for nearly half the proteins in eukaryotes, which includes humans, and viruses. It will provide insight into protein-based illnesses like cancer, type 2 Diabetes and many neurodegenerative diseases, such as Parkinson's disease and Alzheimer's. Just like the early Portuguese explorers that discovered Africa, America and Asia, knowing what we don't know has provided us with a roadmap to focus our future research and agendas. Who knows what secrets are still hidden in the dark proteome.

Supplementary Materials: The datasets generated during and/or analyzed during the current study will be available in the Dark Proteome Database site [<http://darkproteome.ws>];

Author Contributions: NP implemented the Dark Proteome Database, obtained the results, and prepared the manuscript.

Funding: This work was partially supported by Fundação para a Ciência e Tecnologia project UID/EEA/5009/2013.

Acknowledgments: Nelson Perdigão thanks Professor Agostinho Rosa for the validation of the results.

Conflicts of Interest: The authors declare that have conflicts of interest.

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