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How many human proteoforms are there?

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Abstract

Despite decades of accumulated knowledge about proteins and their post-translational modifications (PTMs), numerous questions remain regarding their molecular composition and biological function. One of the most fundamental queries is the extent to which the combinations of DNA-, RNA- and PTM-level variations explode the complexity of the human proteome. Here, we outline what we know from current databases and measurement strategies including mass spectrometry–based proteomics. In doing so, we examine prevailing notions about the number of modifications displayed on human proteins and how they combine to generate the protein diversity underlying health and disease. We frame central issues regarding determination of protein-level variation and PTMs, including some paradoxes present in the field today. We use this framework to assess existing data and to ask the question, “How many distinct primary structures of proteins (proteoforms) are created from the 20,300 human genes?” We also explore prospects for improving measurements to better regularize protein-level biology and efficiently associate PTMs to function and phenotype.

Proteins come in all shapes, sizes and forms. They are deeply involved in the major processes of life and comprise a large and enigmatic space between human genetics and diverse phenotypes of both wellness and disease. Assigning function and dysfunction to proteins is a major challenge for the coming era of basic and clinical research, so we take up the challenge of defining protein composition, including diverse contributions to its variation and the biological ramifications of this diversity.

The size of the human proteome is a matter of debate, and numbers in the literature range from as few as 20,000 to several million[1,2]. The huge discrepancy between these numbers is

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not a scientific controversy, but more a matter of definition. Thanks to the human genome project, we can now estimate the number of protein-coding genes to be in the range of 19,587–20,245 (refs. 1,3,4). Thus, if a single representative protein from every gene is used as the definition of the proteome, the estimated size is just ~20,000. This number may decrease somewhat, as it has been difficult to find an expressed protein encoded by some of these putative protein-coding genes[5,6]. However, if one considers that many genes are transcribed with splice variants, the number of human proteins increases to ~70,000 (per Ensembl[3]). In addition, many human proteins undergo PTMs that can strongly influence their function or activity. These PTMs include glycosylation, phosphorylation and acetylation, among a few hundred others (Fig. 1a), giving rise to many hundreds of thousands of additional protein variants[5]; furthermore, though many proteins are unmodified, some fraction of proteins are already annotated with multiple modifications (Fig. 1b). Finally, selected genes for proteins like immunoglobulins and T-cell receptors undergo somatic recombination to increase the number of potential protein variants into the billions in certain cell types across one's lifetime[7,8].

Each individual molecular form of an expressed protein has come to be called a proteoform[9]. This term captures the disparate sources of biological variation that alter primary sequence and composition at the whole-protein level (Fig. 2). These include biological events that change single or multiple residues within the sequence of amino acids and the many modifications that can decorate the protein during its synthesis or after it is produced within a cell. These sources of variation produce the largely unmapped complexity of human proteoforms. At first glance, characterizing such diversity appears to be intractable, but closer inspection of the sources and limitations imposed upon proteoform diversity, as well as an examination of measurement techniques, can provide bounded estimates. In a few examples, proteoforms and their PTMs have been mapped, enabling early efforts to assign and understand their biological functions.

Sources of proteoform diversity

Our aim is to help diverse communities better understand the composition and nature of human proteins in health and disease. We now assemble known information for the main sources of variation at the levels of DNA, RNA and protein that contribute to proteoform diversity. We then examine how these sources of diversity expand the number of theoretical human proteoforms (Fig. 3a) and contrast that with the number of observed proteoforms carrying multiple PTMs that are actually produced in biological systems (Fig. 3b).

Estimates of DNA-level variation

Substantial sources of variation in human proteins include coding single-nucleotide polymorphisms (cSNPs) and mutations, with ~135,000 validated nonsynonymous cSNPs currently housed within SwissProt. In dbSNP, there are 4.7 million candidate cSNPs, yet only ~670,000 cSNPs have been validated in the 1,000-genomes set as nonsynonymous cSNPs that change the identity of an amino acid in a protein. However, the number of polymorphisms found in databases is reduced to only the two alleles actually harbored by any individual. Our adaptive immune system also presents a major source of somatic

alteration in specific cell types. One can therefore divide these two kinds of variations into ‘population variation’, which exists across the human population, versus ‘individual variation’, which exists in an individual human being.

Main sources of RNA-level variation

Alternative splicing is a key contributor to transcriptome complexity and modulation of complex human traits[10]. RNA sequencing (RNA-seq) studies indicate that ~93% of human genes undergo alternative splicing and about 86% have a minor isoform frequency above 15% (ref. 11). Recently, functional consequences of alternative splicing were explored[12], showing that the interacting partners for minor isoforms from a given human gene are as different as those for proteins encoded by entirely different genes. Alternative splicing often leads to the inclusion or exclusion of mitochondrial targeting sequences, leading to two mature proteins that have identical warheads but are localized to two different places[13]. Transcriptome diversity is further expanded through RNA editing. Though over 4.5 million adenosine- to-inosine editing events (the most common type) have been detected in human transcripts, only ~4,400 actually alter the corresponding amino acid[14].

There is yet a major gap between the number of alternative transcripts asserted by RNA-seq and that detectable by proteomics (e.g., <0.1% of putative novel splice junctions in cancer xenografts)[15]. This discrepancy is due, in part, to the limited sensitivity and coverage of the current proteomic platforms. Although deep proteomic analyses can identify the majority of gene expression, sequence coverage for most proteins remains low, particularly for low-abundance genes. For example, the aggregated NCI-60 proteomics data set[16] covers only 12% of the whole encoded proteome, and only ~5% of the genes had sequence coverage >50% of their protein coding regions[17].

Because small size is a confounding factor in gene prediction, small open reading frames (smORFs) in stretches of RNA previously assumed to be noncoding have only recently been annotated as protein coding regions owing to advances in sequencing and proteomics[18]. Although the total number of novel human small proteins encoded in smORFs is still unclear, with estimates ranging from hundreds to thousands, roles of specific small proteins in fundamental biological processes have been established (for example, control of genome maintenance)[19].

Errors in translation

Errors during protein translation provide one very large source of potential proteome expansion, particularly in aging or stressed cells. Error frequencies of 0.01–0.1% per amino acid (AA) have been estimated for misincorporating structurally similar amino acids *in vivo*[20]. This source of low-level protein heterogeneity is apparent in characterized recombinant proteins expressed in *Escherichia coli*, for which misincorporations can range from 0.5 to 5%. Mistranslation events have also been identified in recombinant monoclonal antibodies expressed in mammalian cell lines, wherein asparagine is substituted for serine at 0.01–0.2% of AGC codons[21].

Post-translational modifications

The exponential increase in the potential number of proteoforms due to PTMs generates an open question in the field that lies at the heart of this Perspective. To help inform and frame the question, one could divide co- and post-translational modifications in different ways (for example, based on their chemical structures or whether or not they are reversible). A structural view would divide PTMs into subtypes based on whether proteins are cleaved or site-specifically modified with 'simple' PTMs (for example, phospho, acetyl, methyl, *O*-GlcNAc, etc.). The structures of other PTMs are highly complex in nature (for example, glycosylation, polyubiquitinylation, etc., as addressed below). The structural view of protein complexity is linked to how PTMs make the number of proteoforms increase and how difficult it is to characterize them precisely. A functional view focuses more on the way that proteoforms and their combinations of PTMs underlie cellular decision making and contribute to overall phenotypes (as has been shown for the histone code[22]).

Complex post-translational modifications

In contrast to the linear assembly of amino acids in polypeptide chains, the ten common monosaccharide building blocks of human glycans can be linked at multiple positions, resulting in highly branched structures. Taking into account the other structural features of oligosaccharides like linear sequence, linkage position and anomeric configuration, the number of possible glycan structures is large. However, one may also conclude that although glycan biosynthesis is untemplated, that does not mean it is unrestricted. Nature may only access a limited number of protein glycoforms. Thus far, on the order of a few tens[23] to more than one hundred[24,25] glycoform compositions above the ~1% detection threshold are readily measured by current technologies, with some examples provided in Table 1.

For ubiquitinylation, homo- and mixed polymers of the 8.5 kDa protein ubiquitin can reach ~25 monomers in length, adding >200 kDa in molecular weight. These modifications exert profound influence on the subcellular location, function and degradation of (apparently) all cellular proteins[26] through complex mechanisms that may include crosstalk with other PTMs (for example, phosphorylation or acetylation). For poly(ADP-ribose), linear chains of 20–50 units combine to form branched polymers that are over 300 residues long. Though a comprehensive understanding of the composition of branched, polymeric PTMs may lie outside immediately available technologies, progress is being made with polyubiquitins, and the growth in native mass spectrometry for high-mass distributions will continue to make inroads and help elucidate the relationship between protein composition, function and disease phenotypes.

How does proteoform number scale with simple PTMs?

The simplistic answer to how PTM number translates to scaling of the number of possible proteoforms is 2^n, where n is the number of PTMs. This refers to site-specific PTMs that are 'binary', like phosphorylation and acetylation (i.e., either on or off). However, consider that a lysine on a protein can exist in at least five different states, taking into account both acetylation and methylation (for example, K_{unmod}, K_{me1}, K_{me2}, K_{me3} and K_{ac}). The general formula describing how proteoform number grows with protein variation is shown in Box 1. A specific example is human histone H4 (UniProt accession: P62805), in which a

combinatorial explosion of its 58 SwissProt-annotated PTMs at 17 known sites gives rise to $>10^{10}$ theoretical proteoforms. Use of just the most common 13 PTM sites from the literature and the E64Q variant (its minor allele frequency is ~0.001%) creates 98,304 possible proteoforms (see Box 1). However, recent analyses of intact H4 proteoforms by seven participating labs reported just 75 proteoforms observed at >0.01% relative abundance[27]. The dramatic, orders-of-magnitude difference between actual and theoretical proteoforms aligns with a view wherein proteoform diversity is limited by a high degree of control over the enzymatic writing and maintenance of PTMs (see section below on proteoform diversity and function).

In protein databases, the number of PTM sites on a single protein can range from 0 to over 90 (see PTM distributions in Fig. 1). Considering only binary modifications makes the number of theoretical proteoforms astronomically large (i.e., $2^{90} = 1 \times 10^{27}$). Here is where two paradoxes arise. The first one is rooted in technologies used to measure protein molecules, whereas the other is one of perspective. Use of technologies that either do, or do not, capture complete compositional information about whole proteoforms drastically changes what is measured and perceived by the scientists using them. Today's perceptions about the diversity of human proteins can be in two extremes: that a majority of the possible variations exist on proteins or that only a minority of possible PTMs actually co-exist on the same protein (see Fig. 2). These different perspectives are central to understanding why protein-level biology is enigmatic, and authors on this Perspective offer a continuum of viewpoints and some data to help frame and inform this open question.

Limits on proteoform diversity

The exponential increase in possible proteoform number due to PTMs creates an explosion in the number of possible protein compositions populated by human biology. There are both natural and technological limits to this 'proteoform explosion', and we deal with each of these in turn.

Copy numbers limit protein complexity in single cells

One limit to protein complexity is copy number. Consider a protein present at 1,000 copies per cell; 1,000 proteoforms is then the maximal number in that cell at a given time. Of course, in a population of 1 million cells, the cell-to-cell diversity could significantly increase that number, especially as cells respond to stimuli by PTM remodeling over time. Such lines of thinking trigger questions regarding how post-translational diversity arose, its function and its range of variation in single states or in response to diverse stimuli.

Handling the proteoform explosion via abundance thresholding

Another valuable point of reference comes from consideration of just how many genes are expressed into a protein in a given cell type. Estimates from deep proteomics and transcript profiling suggest that about half the human genome is expressed in proteins at over 20 copies per cell in a given cell type (i.e., about 10,000 of the 20,000 human genes)[28]. Assuming this expression threshold of 10,000 genes and allowing for detection of ~100 proteoforms for each gene product, one then multiplies these two to arrive at a measurement

target of 1,000,000 distinct proteoforms in a given cell type. A 2016 estimate based on trends in databases indicated that the number may be ~6 million proteoforms[29]. Better estimates of this proteoform diversity are needed, and are analogous to the extrapolations of the number of human genes using expressed sequence tags (ESTs) in the year 2000 (ref. 30).

The question of how many proteoforms exist may prove impossible to answer fully (i.e., down to single copy proteins or across a billion-fold dynamic range of the most-to-least-abundant proteoforms). Errors in transcription and translation or exposure to toxic chemicals can produce numerous low-abundance proteoforms, perhaps even at the single-molecule level in a large population of cells. However, this issue may be more philosophical than practical, as current technologies for identifying and tracking proteoforms (for example, chromatography, mass spectrometry and antibody-based measurements) are constrained to operate above a given abundance level (i.e., the number of detectable proteoforms rather than all proteoforms *per se*). Through this lens, the number and variety of proteoforms expressed in biological systems appears to be well below the theoretical combinatorial possibilities[31], with several examples providing a glimpse into this open question (Table 1). However, should new technologies emerge that relieve these constraints (for example, single-molecule proteoform detection[32]), this comfortable myopia may prove fragile.

Challenges in measuring proteoforms

Inference versus direct readout of proteoforms

The dominant paradigm of modern proteomics is the ‘bottom-up’ strategy, in which protein mixtures are digested with a protease, typically trypsin, to yield complex mixtures of peptides (Box 2). These peptides are analyzed by LC–MS/MS and identified by comparison of their MS/MS fragmentation spectra with theoretical spectra produced from the known genome sequence of the organism under study or customized protein sequence databases derived from matched DNA- or RNA-sequencing data from the same sample. The presence of a given protein in the sample is inferred from identification of the peptides it contains, in a process known as ‘protein inference’[32]. Although protein inference is a widely employed cornerstone of bottom-up proteomics, it is not generally possible to identify proteoforms in the same manner, as different proteoforms often share most of their peptides with one another. Instead, it is necessary to use ‘top-down’ proteomic methods, in which the entire proteoform is analyzed by LC–MS/MS without prior digestion to peptides (Box 2). Ideally, the complete amino acid sequence and localized PTMs are obtained; for proteins that are especially large or those harboring many PTMs, there are often ambiguities in the complete description of related proteoforms. Addressing these limitations of top-down proteomics in both denatured and native modes is a frontier area of current research.

Mapping protein composition with complete molecular specificity

The next stage of proteomic investigation goes beyond identification of peptides and individual PTMs to reach for complete protein characterization through proteoform-resolved measurement[34,35]. For elucidating functions of proteoforms, complete knowledge of their molecular composition and that of their interacting partners is preferred. A potential confounding factor in this endeavor can arise from artifacts of sample preparation of tissues,

cells and their extracts via enzymatic or chemical modification or degradation (for example, oxidation and chain cleavage). As a result, proteoforms can be proteolytically truncated, thereby forming new proteoforms with a loss of correlative power and relationship to their function. Enzymatic conversions of proteoforms also occur in body fluids *in vitro*, further complicating their quantification. For example, brady-kinin, a proteoform of kininogen-1 and a vasoactive peptide hormone, is degraded faster *in vitro* than *in vivo* (half-life of 17 s). In addition, other enzymes like phosphatases can convert proteoforms in homogenates unless they are inhibited. With respect to these problems, new sampling procedures like direct mass-spectrometric imaging[36,37] of tissues yielding the spatial distribution of proteoforms and Picosecond InfraRed Laser technology (PIRL) are promising for providing higher fidelity readouts of whole proteoforms[38]. Tissue samples collected with PIRL by cold, soft and very fast ablation show more intact proteoforms than those obtained by conventional protein extraction[39].

Prospects for mapping the majority of human proteoforms

With proteins being dynamic and so dependent on their context, it is critical to frame the dimensions of their measurements. Analysis of protein molecules can be performed at different levels in the hierarchical organization of the human body (Fig. 4). Mapping of proteins can also mean determining their spatial distribution in a solid tissue or deducing their atom composition. The question arises as to what level of understanding is needed to obtain a holistic view of the human proteome and how that would augment our biomedical goals for science, technology and society. Recent efforts to describe the composition and spatial distribution of proteins have advanced in draft maps of the human proteome[40,41] and the Human Protein Atlas[2], respectively. This year, a major endeavor called the Human Cell Atlas has been launched to define the cell types that comprise the human body[42]. This effort will expand with a variety of consortia and take on the definition of cell types in diverse organs, the immune system of the blood and bone marrow, and even the brain.

How much proteoform variation exists between cell types?

Recent advances in single-cell RNA sequencing (scRNA-seq) technology allow comprehensive and data-driven characterization of major cell types within a tissue[42]. For some tissues, estimates based upon the sum total of previous (pre-single-cell) studies provide a good estimate of the number of cell types, whereas for other tissues there are many cell types that remain to be classified. One could envision that analysis of cellular proteoforms would complement the scRNA-seq gene expression data and add power to robust classification of cell types and states. Additionally, with the availability of the Human Cell Atlas, an effort focused on compositional mapping of proteoforms in each human cell type could become feasible, as outlined in a separate publication[43]. This cell-based approach to compositional mapping of human proteins was framed for a depth of 250,000 proteoforms per cell type[43], with a focus on defining normal variation in health and wellness; such a project would require establishing cost effective approaches to cell- and proteoform-specific measurements.

Mapping proteoforms and their kinetics in health and disease

With the overview above regarding sources of combinatorial variation, what are some functional implications arising from this protein-level diversity? Once a PTM present only on a specific splice variant can be asserted precisely, how does it vary across cell type and disease? Such questions are being addressed using a common approach of mapping proteoforms, determining their composition (including any new ones resulting from mutation or aberrant PTM patterning), and then correlating proteoform-level dynamics to functional readouts and phenotype (Fig. 5). Several examples from the past few years are summarized in Table 1, with reviews available to highlight early examples[44]. In the domain of microbiology and infectious disease, the process of assigning proteoform function and obtaining clinical value is farthest along. In more complex human diseases across the spectrum of neurodegeneration, oncology and cardiovascular disease, functional assignment for combinations of events detected at the proteoform level are accruing, albeit at a slower rate.

In cancer epigenetics, there are several examples in which PTM crosstalk has been mapped definitively (see also the top rows of Table 1)[22,45–49]. It has been estimated that ~1,000 H3 proteoforms above a 0.1% abundance threshold exist for each of three histone H3 genes[50]. Such examples have been mapped in the context of multiple myeloma[51] and diffuse intrinsic pontine glioma (DIPG)[52]. In each case, a global decrease in trimethylation of histone H3 on lysine 27 (H3K27me3; normally ~20% abundance) could result in hundreds of dysregulated histone codes in the diseased epigenome. In other disease areas, including organ fibrosis, several examples exist in which a mutation at one site can affect PTM profiles elsewhere on the protein[53–55]. In neurological disease and aging, modified proteins are the histopathological hallmarks of a number of diseases, such as SOD1 in amyotrophic lateral sclerosis (ALS)[56,57], and a class of diseases long referred to as the proteinopathies[57], including tauopathies in Alzheimer's disease[58] and inclusions of amyloid-β[59], α-synuclein in Parkinson's[60,61] and multiple secondary ubiquitinopathies[62,63]. In heart disease, proteoform dynamics have been observed on proteins such as cardiac troponin I[64], apolipoprotein C-III[65], and B-type natriuretic peptide, the latter a key regulator of blood pressure and also the gold standard biomarker for clinically assessing heart failure[66]. Within the field of infectious diseases, proteoform-resolved approaches have been instrumental for understanding infectivity and dissemination of *Salmonella typhimurium*[67], *Corynebacterium glutamicum*[68] and *Neisseria meningitidis*[69]. Finally, the clinically deployed use of whole-protein MALDI–TOF MS for rapid identification of the species and strain of pathogenic bacteria has been adopted by thousands of hospitals and clinics worldwide[70,71].

Deciphering the functions of proteoforms and their PTMs

With a far more precise understanding of protein composition and distribution in human biology, several advances can be anticipated. For compositional proteomics, the assignment of proteoform function and their combinations of PTMs can be made more efficient, as this is a holy grail in both basic and translational research. The use of proteoforms as protein-based biomarkers of disease is in its infancy (Table 1). To assign biological functions to

proteoforms and their PTMs, a more precise map of protein composition would be the basis for the creation of new reagents and tools, such as the two examples outlined below.

Proteoform synthesis for functional studies

Understanding the language of site-specific PTMs remains a challenge, in part, because endogenous proteins are complex mixtures of related compositions, depending on their biosynthesis, functional regulation and subcellular distribution. Tools and technologies for precision proteoform synthesis (i.e., the ability to produce useful quantities of proteins with defined post-translational decorations for biochemical, mechanistic and structural studies) have advanced recently via two main approaches. First, the installation of genetically encoded chemistry by co-translationally incorporating noncanonical amino acids site specifically into proteins has afforded new advances (for example, phosphorylated amino acids)[72–74]. Furthermore, precision installation of glycans affords chemically defined glycoforms to study their structure and function. Recent efforts in glycoengineering of cellular systems have also expanded our ability to reliably synthesize chemically defined glycoforms[75]. Complementing these cell-based strategies (and emerging cell-free alternatives[76]) are well-established protein chemical synthesis and semisynthesis strategies for preparing proteoforms containing a wide repertoire of PTMs[77]. For example, histone proteoforms harboring multiple PTMs have been generated for functional studies via semisynthesis.

Affinity reagents and assays

The need to understand and assert PTM function benefits from antibody and mass-spectrometric methods working in a complementary and proteoform-informed fashion. For the development of affinity reagents, full-length proteoforms or domains decorated with PTMs are needed as antigens for production and validation of high-quality affinity reagents using methods like phage display[78]. The use of multiple antibodies, created using full-length antigens, can be deployed for cell-type-resolved or spatial mapping using frontier methods like mass cytometry (CyTOF) or for targeted analysis of a few dozen epitopes using single-cell proteomics[79]. Combining these methods with proteoform information by creating affinity reagents based upon precise knowledge of protein composition would enable efforts to map the spatial information of proteoforms in distinct cell types within human tissues. In the long-term, it is crucial to generate recombinant antibody tools as monospecific, permanent and renewable reagents to replace perishable animal-derived polyclonal or even monoclonal antibodies. Moreover, to detect proteins in their natural state, it is important that recombinant antibodies be generated to intact and folded proteins, because most high-affinity and specific antibodies recognize tertiary, not primary, sequence determinants. To this end, the National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium's (NCI–CPTAC) Antibody Portal (http://antibodies.cancer.gov) provides well-characterized, renewable antibodies against full-length protein antigens that are useful in development of targeted assays (e.g., immuno-MRM; http://assays.cancer.gov).

On the roles of PTM complexity in human biology

Below, we provide some thoughts on the possible roles of proteoform diversity, first through the evolution of complex traits and then on information processing for complex systems like

human cells. Although the potential complexity afforded to proteins by PTMs is enormous, the few studies available suggest that only a small amount of this complexity is accessed in any given biological context. At the same time, different contexts may elicit diverse parts of proteoform complexity, so large swaths of PTM combinatorial space could have been explored over evolutionary timescales. One way to think about the role of this complexity is that it offers an 'escape' from the central dogma by accessing a far broader ensemble of protein compositions than can be realized from the genetic level alone[80]. PTMs thereby create capabilities that would not be accessible through protein translation or diverse splicing mechanisms. This viewpoint interprets PTMs as providing protein conformational states that may subsequently be exploited to modulate effector pathways in response to physiological conditions. For instance, PTM codes on central hub proteins like histones can be seen as a form of molecular 'weak linkage', which can facilitate evolution of higher complex traits[81].

Another perspective is that biological systems select for proteoform diversity to improve robustness by having a distribution of forms and activities. This point of view actually argues for some level of promiscuity of PTM transferases in creating proteoform diversity. Natural selection must constantly wrestle with the tradeoff between fidelity and variability. High fidelity of biochemical processes would seem, at first blush, to be advantageous for a tightly orchestrated biological system; however, the higher the fidelity, the more amenable the organism is to the mutation and selection processes that are central to evolution. Moreover, although natural selection cannot look ahead, variation within proteins in a population allows more opportunities for later exploitation and adaptation as selective conditions change.

Protein compositions play a central role in cellular information processing[47]. For information 'coding' in cellular signaling, the 'histone code' is perhaps the best-known example[22]. It is usually implied that some highly modified proteins can act as hubs to integrate signals and orchestrate complex cellular functions[82]. In this area of chromatin biology, individual PTMs are sometimes called 'marks'; combinations of PTM marks make up 'codes' (which are captured through proteoform measurement). Different combinatorial patterns of PTMs are 'written' on these hubs by the combined activity of forward-modifying and reverse-demodifying enzymes in response to varied physiological conditions. This framework is being extended to other PTM marks like phosphorylation, methylation, acetylation, ubiquitination, etc., and they combine to regulate responses to physiological conditions[80] and to fine-tune individual molecular interactions. Such interactions can themselves be formidably intricate. More than 5% of the protein complement in a cell are enzymes (for example, ~500 kinases and ~140 protein phosphatases are encoded in the human genome), which can both compensate for and compete with each other at individual amino acids (for example, O-GlcNAcylation on canonical phosphorylation sites) and PTMs can be clustered in 'hotspots' at which different PTMs can influence each other. The resulting combinatorial patterns of PTMs convey information through 'PTM crosstalk' on the protein. The resulting PTM codes can then be 'read' by downstream interacting proteins in effector pathways. In this way, multiple upstream processes can collectively orchestrate a variety of downstream processes in various ways depending on conditions while working through one or a few hub proteins. The tumor suppressor p53, which can be modified on

over 100 sites[47], serves as a clear example of this case; however, what is unknown is how many of these PTMs coexist on the same proteoform.

Whether in the context of human evolution or cellular signaling, this tension arises for proteoforms: are PTMs and other differences between proteoforms carefully controlled and regulated or are they subject to high levels of stochastic 'noise'? The answer may well be both, with different strategies being appropriate in different cellular or developmental contexts. For histones, a relatively strict doctrine of PTM writing and maintenance appears to limit the combinatorial explosion of proteoforms. Outside of histone biology, it is not well known whether systems for protein-based coding in the language of PTMs are prone to 'loose constructionism', defined here as a high tolerance for imprecision and noise in writing and erasing PTMs in cells of a living organism. How much of each strategy is operative and in which contexts? For unravelling such questions regarding the fidelity of information encoding and transfer, it will be essential to quantify the distribution of modification patterns on proteins, to develop mathematical and statistical frameworks for analyzing these distributions and to experimentally demonstrate how protein 'readers', 'writers' and 'erasers' interact with these distributions. Addressing these challenges in distinct areas of biology, even with improved tools for precise determination of protein composition at the proteoform level, will take several years to sort out[47].

Summary and future prospects

From the many considerations above, a precise estimate of the number of human proteoforms is still difficult to provide. Finding ways to sample and better estimate proteoform number would assist in bounding the breadth and depth of the human proteome. For a given cell type, the depth of proteome coverage needed to detect the majority of human proteoforms above a specified threshold can serve as a protein-level analog of the 5× genome coverage employed for sequencing the first human genomes. For example, the 1,000,000 proteoform mark for cells of a given type would allow for mapping of ~100 proteoforms for each expressed gene. Compositional proteomics is maturing to the point whereby such depth may become possible to better decipher conserved, functional PTMs relative to biochemical noise. At whatever depth, building proteoform-informed measurement modalities to translate absolute molecular knowledge for proteins (and their combinatorial sources of modification) into deep functional insight will assist efforts to regularize and even domesticate the human proteome in the years ahead. Whether a large-scale endeavor to compositionally map cellular proteomes is launched depends on the perceived feasibility, endpoint(s) and value of such a project, and we hope this Perspective allows diverse communities to better frame the open questions about the composition and nature of the human proteome in both health and disease.

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Box 1

Calculating the number of theoretical proteoforms

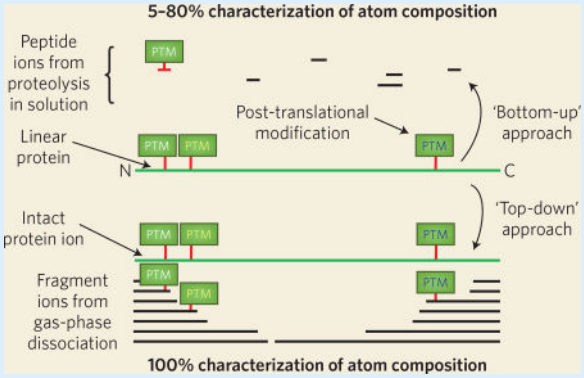
$$\#\ \text{theoretical proteoforms} = \prod_{i=1}^{n} (\text{potential PTMs at } \text{AA}_i + 1)$$

Recent analyses of human histone H4 (P62805) mapped 75 proteoforms in human cells (see main text). Considering the 13 most common PTMs (acetylation, methylation and phosphorylation, shown below) and a single SNP, 98,304 theoretical proteoforms are possible.

2^6 (K5/8/12/16/31/91 ac) × 3^1 (R3 me1/2) × 4^1 (K20 me1/me2/me3) × 2^5 (S1/S46/Y51/T79/Y87 ph) × 2 (N-term ac) × 2 (E63Q cSNP) = 98,304 proteoforms

Box 2

Bottom-up and top-down strategies for the analysis ofprotein sequence and composition



With the ‘bottom-up’ proteomics workflow, preanalytical processing of proteins (and corresponding proteoforms) is performed with proteases (e.g., trypsin) to generate analytically manageable peptides (top) that are sequenced in order to determine protein identity. The ‘top-down’ approach avoids the digestion step and characterizes proteoform microheterogeneity directly through tandem mass spectrometry techniques (bottom). Analysis at the intact level is advantageous because 100% of the proteoform’s primary structure is present in the top-down workflow, contrasting with bottom-up methods in which incomplete sampling of peptides across the protein backbone may cloud actual proteoform determination.

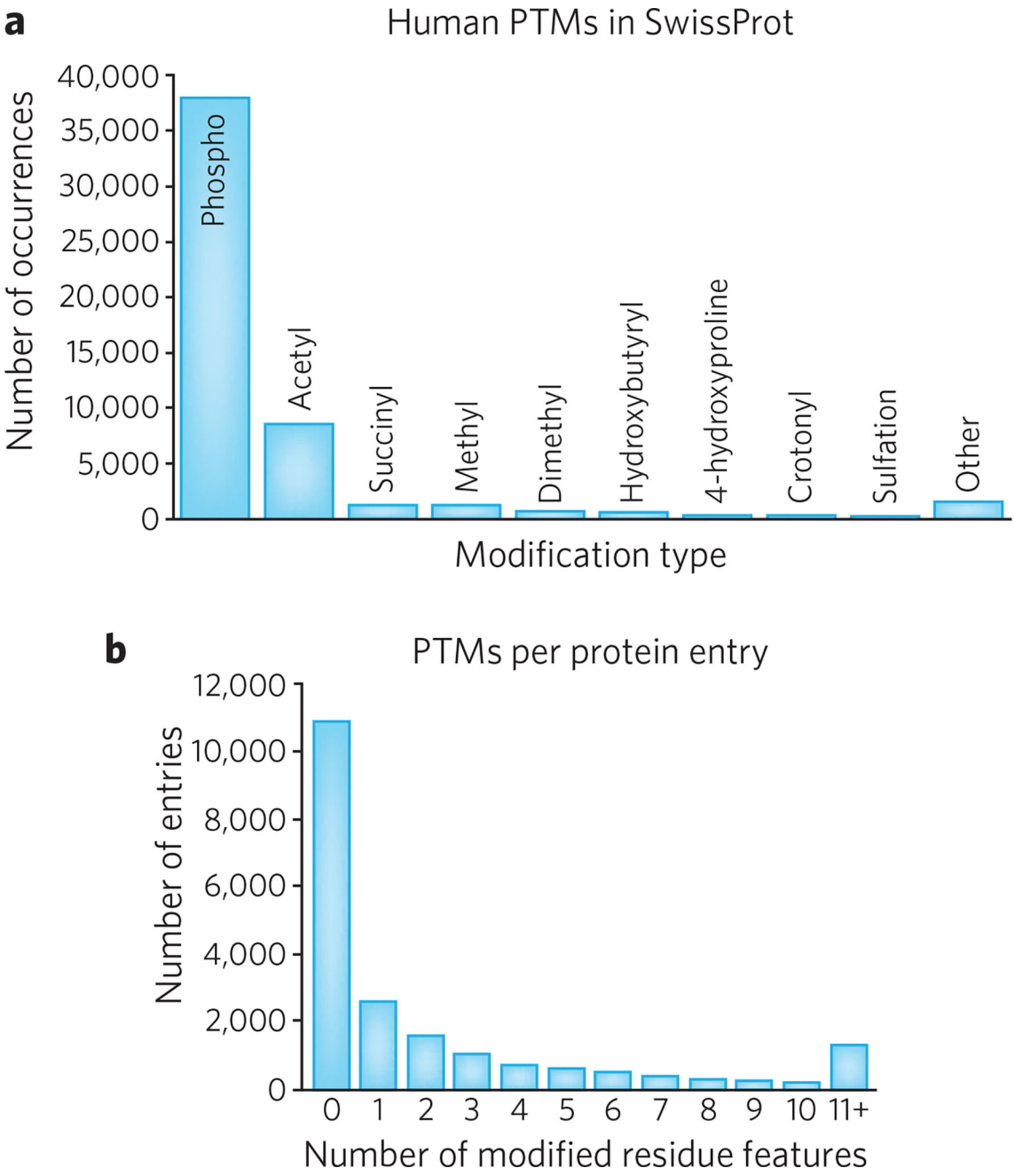


Figure 1. Two parsings of post-translational modifications from the SwissProt database of 20,245 human proteins

(**a**) Histogram of PTMs in SwissProt for *Homo sapiens* (taxon identifier: 9606). Phosphorylation (phospho) is by far the most frequently annotated PTM at 38,030 (72%). Note that there are ~400 different types of PTMs known in biology (see: http://www.unimod.org). (**b**) Histogram of PTMs per SwissProt entry. Note that the distribution of PTMs is not uniform with 75% of entries containing two or fewer annotated PTMs; yet only five entries have >90 annotated PTMs.

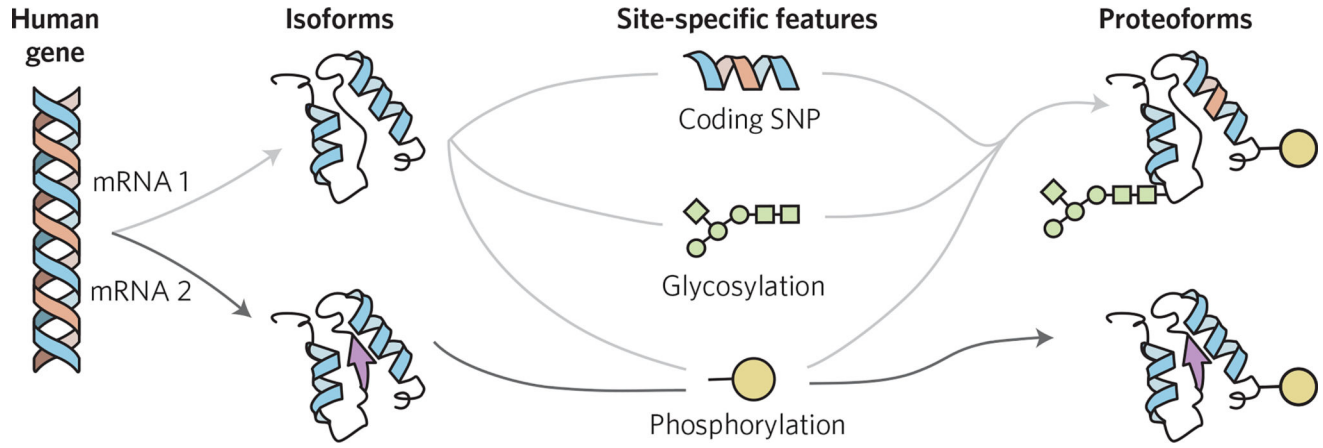


Figure 2. Graphical depiction of sources of protein variation that combine to make up proteoforms, each of which map back to a single human gene

Depicted is a single human gene and two of its isoforms, which differ by the coding for several different amino acids of a protein primary sequence (at left); isoforms commonly arise from alternative splicing of RNA and from use of different promoters or translational start sites. Isoform variation combines with site-specific changes to generate human proteoforms (at right); three examples of site-specific changes include single-nucleotide polymorphisms (SNPs) and co- or post-translational modifications like N-glycosylation or phosphorylation, respectively.

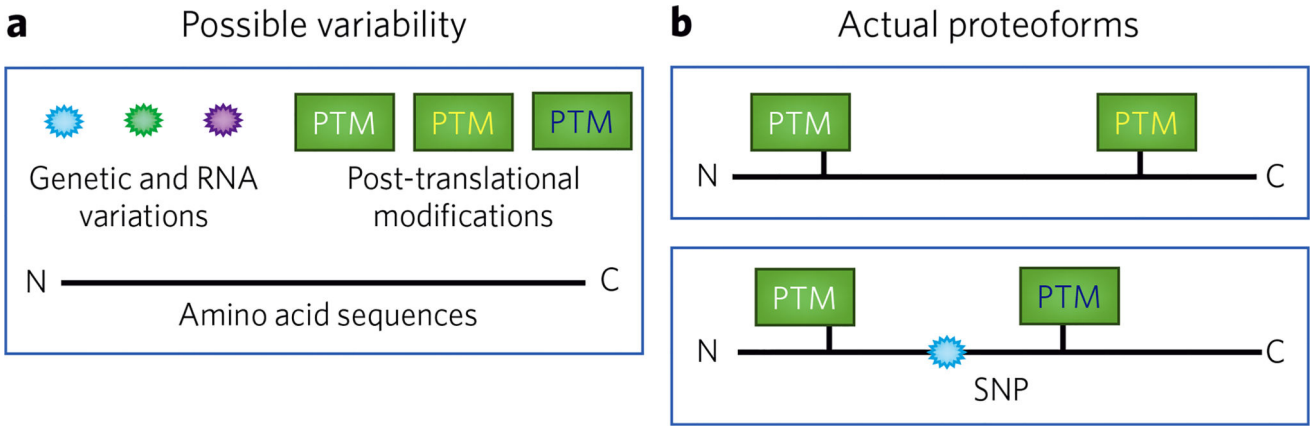


Figure 3. Contrasting the potential sources of protein variability versus those that actually occur in combination as proteoforms detectable in actual human systems

(**a**) Common sources of protein variability include alternative splicing of RNA, single-nucleotide polymorphisms (SNPs) in regions of genes coding for amino acids, and PTMs. Note that there are ~33,000 splice isoforms, ~78,000 site-specific amino acid variants (i.e., polymorphisms and mutations) and ~53,000 PTMs in the October 2017 release of the Human SwissProt database. (**b**) Depiction of two proteoforms from specific combinations of protein variability.

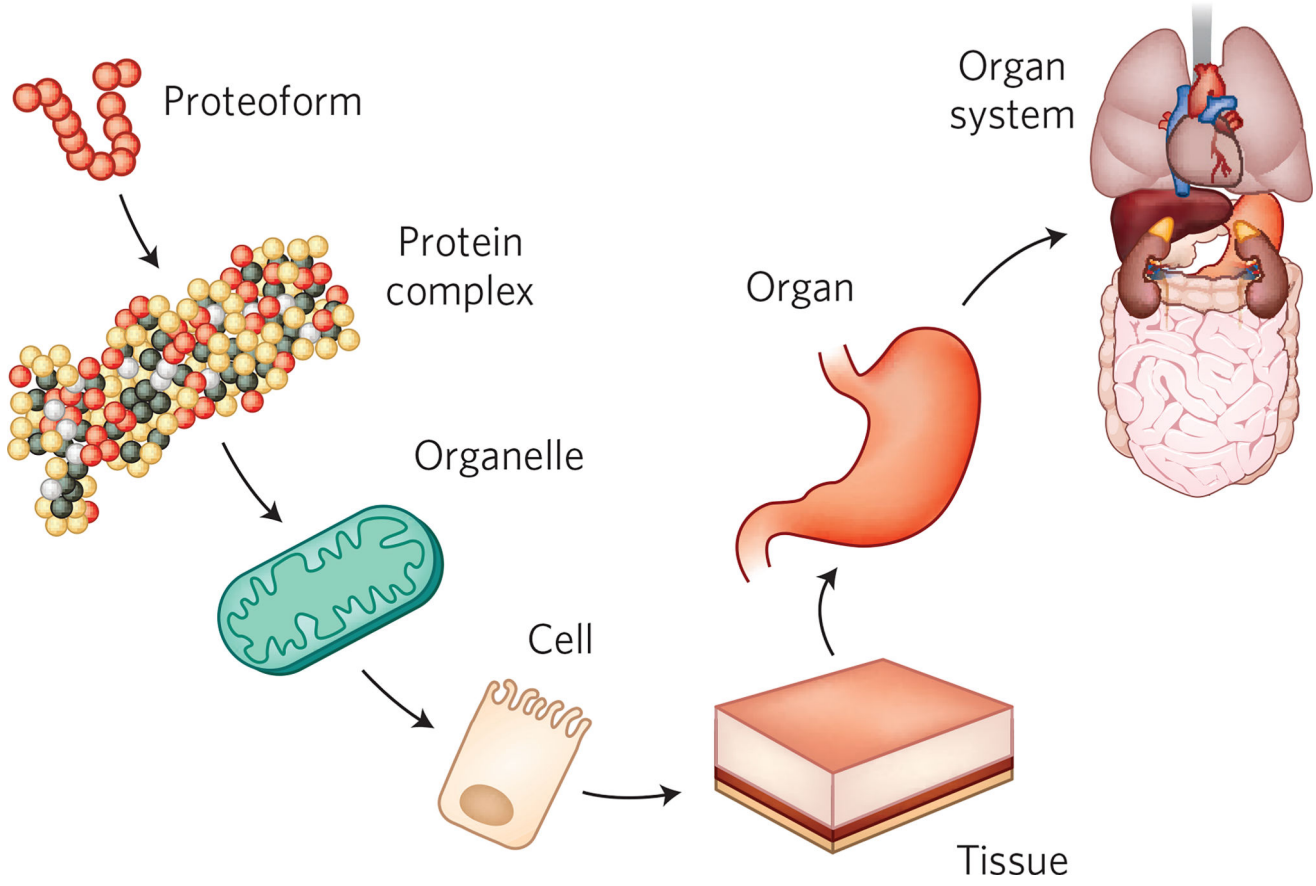


Figure 4. Levels of organization in the human body

Starting from protein primary structure (proteoforms), the complexity of organ systems is built up in layers. A key concept is that diverse measurement approaches in proteomics seeks analysis of protein molecules at the various levels and contexts represented. Proteoform membership in protein complexes and localization within organelles, cells and tissues are all aspirations of measurement technologies to map protein molecules more precisely in molecular composition, across space and through time.

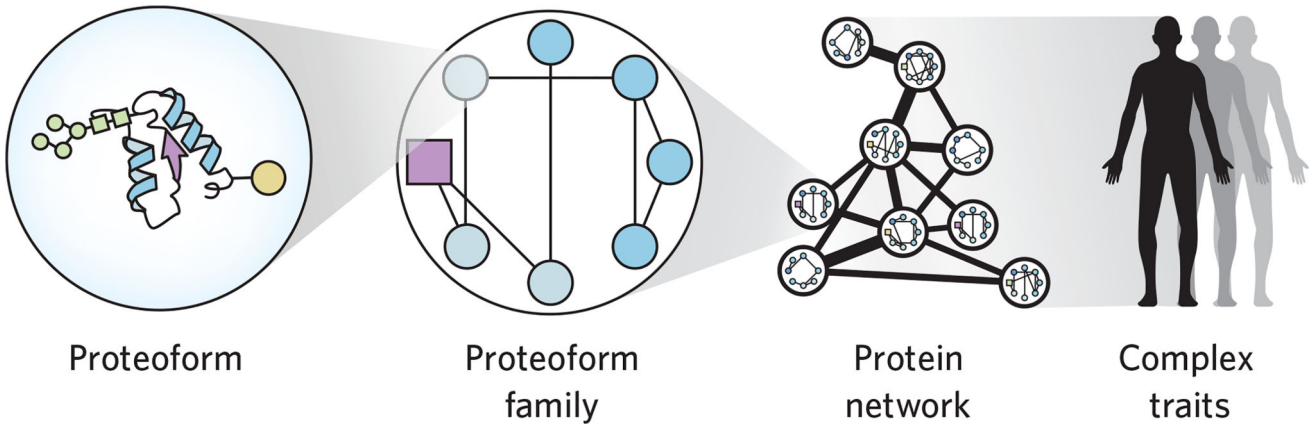


Figure 5. Proteoforms and their families underlie complex traits and molecular mechanisms operative in living systems

In nature, individual proteoforms (left), arising from variable sources of biological variation like PTMs, often exist in groups of related proteoforms. These dynamic 'proteoform families' (middle left) are the true protein products from the same human gene that convey information within signaling and regulatory networks (middle right) that underlie complex traits in wellness and disease (right). Discrete proteoforms and their families offer challenging, high-value targets for direct measurement by top-down proteomics.

Table 1

Examples of mapping proteoforms and correlating them to function and phenotype in complex systems

System	Number of proteoforms mapped	Proteoform→PTM→function
14-3-3 proteins[83]	11[a]	Phosphorylation mediates protein–protein interaction
α-Synuclein; human brain in Parkinson's disease[60]	11	Phosphorylation→weak correlation to Parkinson's disease pathology
Amyloid-β; human brain in Alzheimer's disease[59]	23	Diversity of proteoforms is not captured by traditional ELISA assays
Angiotensin converting enzyme; human[84]	24[a]	Multiple isozymes with multiple functions
Apolipoprotein C-III; human high-density lipoprotein particles[65]	4	Branched glycoproteoforms on Thr104 correlate with HDL-C levels
B-type natriuretic peptide; heart failure[66,85]	7–24	Multiple PTMs and proteolysis correlate with heart failure
Cardiac troponin I; heart failure[64,86]	17	Altered in phosphoproteoforms associated with cardiac disease
Chorionic gonadotropin; α/β subunits, human[23]	10 and 24[b]	Sialic acid content influences receptor binding activity and clearance
Erythropoietin; recombinant fusion protein expressed in CHO cells	>230[c]	Modulation of receptor binding kinetics during red blood cell production
Etanercept; human[24]	>80	Galactosylation and fucosylation modulate immunogenic potency
Histone H2B; human[46]	15	Many gene family members possible→few observed proteoforms
Histone H3; human[87]	>250[d]	Low dosage of H3.3K27M (<10%) associated with pediatric diffuse intrinsic pontine gliomas (DIPG)[52]
Histone H4; human[27,88]	75	Associated with both gene repression and activation
Interferon β-1a; commercial recombinant protein (Avonex)[89]	138	Loss of N-terminal Met correlated with multiple sites of deamidation and loss of potency (used clinically to treat multiple sclerosis)
Myosin regulatory light chain; swine heart failure[90]	4	Decreased phosphorylation correlates with myocardial infarction
Outer membrane proteins in *C. glutamicum*[68]	30	O-mycoloylation→localization to the outer membrane
PilE, pilin proteins in *N. meningitidis* infection[69]	18	Phosphoglycerylation→increased *in vivo* dissemination and virulence
Reactive cysteines in *S. typhimurium* infection[67]	34	S-glutathionylation and S-cysteinylation→infection-like conditions
Transthyretin; familial amyloidosis[55]	25	Genetic mutation alters PTM profiles

Proteoforms and their PTMs have been mapped on selected microbial, pig, mouse and human proteins.

[a] Estimated.

[b] Human chorionic gonadotropin (hCG) is another prominent example for which glycosylation strongly regulates its biological function. The hCG protein contains more than 40 N- and O-glycan structures on two glycosylated subunits. Combinatorial analysis for the α-subunit and β-subunit predicts ~16,000 theoretical glycoforms; however, only 10 and 24 could be assigned for each subunit, respectively.

[c] Although glycosylation is an untemplated process, when one takes into account multiple functional and biosynthetic arguments, it has been estimated that fewer than 3,000 N- and O-linked glycan monomers exist in humans[91].

[d] Recent estimates from middle-down studies suggest that ~1,000 proteoforms exist for each of the H3 genes[50,92].