

1 **Analysis of protein-coding genetic variation in 60,706 humans**

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1 **Summary**

2 Large-scale reference data sets of human genetic variation are critical for the medical
3 and functional interpretation of DNA sequence changes. Here we describe the
4 aggregation and analysis of high-quality exome (protein-coding region) sequence data
5 for 60,706 individuals of diverse ethnicities. The resulting catalogue of human genetic
6 diversity has unprecedented resolution, with an average of one variant every eight bases
7 of coding sequence and the presence of widespread mutational recurrence. The deep
8 catalogue of variation provided by the Exome Aggregation Consortium (ExAC) can be
9 used to calculate objective metrics of pathogenicity for sequence variants, and to identify
10 genes subject to strong selection against various classes of mutation; we identify 3,230
11 genes with near-complete depletion of truncating variants, 79% of which have no
12 currently established human disease phenotype. Finally, we show that these data can be
13 used for the efficient filtering of candidate disease-causing variants, and for the
14 discovery of human “knockout” variants in protein-coding genes.

15

16 **Background**

17 Over the last five years, the widespread availability of high-throughput DNA sequencing
18 technologies has permitted the sequencing of the whole genomes or exomes (the
19 protein-coding regions of genomes) of over half a million humans. In theory, these data
20 represent a powerful source of information about the global patterns of human genetic
21 variation, but in practice, are difficult to access for practical, logistical, and ethical
22 reasons; in addition, the inconsistent processing complicates variant-calling pipelines
23 used by different groups. Current publicly available datasets of human DNA sequence
24 variation contain only a small fraction of all sequenced samples: the Exome Variant
25 Server, created as part of the NHLBI Exome Sequencing Project (ESP)¹, contains
26 frequency information spanning 6,503 exomes; and the 1000 Genomes (1000G) Project,
27 which includes individual-level genotype data from whole-genome and exome sequence
28 data for 2,504 individuals².

29

30 Databases of genetic variation are important for our understanding of human population
31 history and biology¹⁻⁵, but also provide critical resources for the clinical interpretation of
32 variants observed in patients suffering from rare Mendelian diseases^{6,7}. The filtering of
33 candidate variants by frequency in unselected individuals is a key step in any pipeline for
34 the discovery of causal variants in Mendelian disease patients, and the efficacy of such

1 filtering depends on both the size and the ancestral diversity of the available reference
2 data.

3

4 Here, we describe the joint variant calling and analysis of high-quality variant calls
5 across 60,706 human exomes, assembled by the Exome Aggregation Consortium
6 (ExAC; exac.broadinstitute.org). This call set exceeds previously available exome-wide
7 variant databases by nearly an order of magnitude, providing unprecedented resolution
8 for the analysis of very low-frequency genetic variants. We demonstrate the application
9 of this data set to the analysis of patterns of genetic variation including the discovery of
10 widespread mutational recurrence, the inference of gene-level constraint against
11 truncating variation, the clinical interpretation of variation in Mendelian disease genes,
12 and the discovery of human “knockout” variants in protein-coding genes.

13

14 **Variant discovery and quality control**

15 Details of the variant calling process are provided in Supplementary Information. Briefly,
16 we assembled approximately 1 petabyte of raw sequencing data (FASTQ files) from
17 91,796 individual exomes drawn from a wide range of primarily disease-focused
18 consortia (Supplementary Information Table 2). We processed these exomes through a
19 single informatic pipeline and performed joint variant calling of single nucleotide variants
20 (SNVs) and short insertions and deletions (indels) across all samples using a new
21 version of the Genome Analysis Toolkit (GATK) HaplotypeCaller pipeline
22 [Supplementary Information Section 1.3; Banks *et al.*, in preparation]. At each site,
23 sequence information from all individuals was used to assess the evidence for the
24 presence of a variant in each individual. We also performed systematic analysis of copy
25 number variation across these individuals [Ruderfer *et al.*, to be co-submitted].

26

27 We leveraged a variety of sources of internal and external validation data to calibrate
28 filters and evaluate the quality of filtered variants (Supplementary Information Table 6).
29 We adjusted the standard GATK variant site filtering⁸ to increase the number of singleton
30 variants that pass this filter, while maintaining a singleton transmission rate of 50.49%,
31 very near the expected 50%, within sequenced trios. We then used the remaining
32 passing variants to assess depth and genotype quality filters compared to >10,000
33 samples that had been directly genotyped using SNP arrays (Illumina HumanExome)
34 and achieved 97-99% heterozygous concordance, consistent with known error rates for

1 rare variants in chip-based genotyping⁹. Relative to a “platinum standard” genome
2 sequenced using five different technologies¹⁰, we achieved sensitivity of 99.8% and false
3 discovery rates of 0.13% for single nucleotide variants (SNVs), and corresponding rates
4 of 95.1% and 1.95% for insertions and deletions (indels).

5

6 In order to generate allele frequencies based on independent observations without
7 enrichment of Mendelian disease alleles, we restricted the final release data set to
8 unrelated adults with high-quality sequence data and without severe pediatric disease;
9 full details of the filtering process are described in the Supplementary Information. After
10 filtering, the final ExAC data set comprises 60,706 individuals (Figure 1a). To identify the
11 ancestry of each ExAC sample, we performed principal component analysis (PCA) on
12 5,400 common SNVs with high coverage across all of the exome capture technologies¹¹
13 represented in ExAC. This PCA allows us to distinguish the major axes of geographic
14 ancestry within the ExAC sample, and to identify population clusters corresponding to
15 individuals of European, African, South Asian, East Asian, and admixed American
16 (hereafter Latino) ancestry (Figure 1b; Supplementary Information Table 3). We further
17 separated Europeans into individuals of Finnish and non-Finnish ancestry given the
18 enrichment of this bottlenecked population; the term “European” hereafter refers to non-
19 Finnish European individuals.

20

21 We identified 10,195,872 candidate sequence variants in ExAC. We further applied
22 stringent depth and site/genotype quality filters to define a subset of 7,404,909 high
23 quality (HQ) variants, including 317,381 indels (Supplementary Information Table 6),
24 corresponding to one variant for every 8 bp within the exome calling intervals. The
25 majority of these are very low-frequency variants absent from previous smaller call sets
26 (Figure 1c): of the HQ variants, 99% have a frequency of <1%, 54% are singletons
27 (variants seen only once in the data set), and 72% are absent from both 1000G and
28 ESP.

29

30 However, the density of variation in ExAC is not uniform across the genome, and the
31 observation of variants depends on factors such as mutational properties and selective
32 pressures. In the ~45M well covered (80% of individuals with a minimum of 10X
33 coverage) positions in ExAC, there are ~18M possible synonymous variants, of which
34 we observe 1.4M (7.5%). However, we observe 313K out of 499K (62.8%) of possible

1 CpG transitions (C to T variants, where the following base is G), while only observing
2 294K out of 9.3M transversions (3.1%) and 802K out of 8.9M other transitions (9%). A
3 similar pattern is observed for missense and nonsense variants, with lower proportions
4 due to selective pressures (Figure 1D). Of 123,629 HQ indels called in coding exons,
5 117,242 (95%) have length -6 to +6, with shorter deletions being the most common
6 (Figure 1E). Frameshifts are found in smaller numbers and are more likely to be
7 singletons than in-frame indels (Figure 1F), reflecting the influence of purifying selection.

8
9

10 **Patterns of protein-coding variation revealed by large samples**

11 The unprecedented density of protein-coding sequence variation in ExAC reveals a
12 number of properties of human genetic variation undetectable in smaller data sets. For
13 instance, 7.9% of HQ sites in ExAC are multiallelic (multiple different sequence variants
14 observed at the same site), close to the Poisson expectation of 8.3% given the observed
15 density of variation, and far higher than observed in previous data sets - 0.48% in 1000
16 Genomes (ExAC calling intervals) and 0.43% in ESP.

17

18 The size of ExAC also makes it possible to directly observe mutational recurrence:
19 instances in which the same mutation has occurred multiple times independently
20 throughout the history of the sequenced populations. For instance, among synonymous
21 variants, a class of variation expected to have undergone minimal selection, 43% of
22 validated *de novo* events identified in external datasets of 1,756 parent-offspring trios
23 are also observed independently in our dataset (Figure 2a), indicating a separate origin
24 for the same variant within the demographic history of the two samples. This proportion
25 is much higher for transition variants at CpG sites, well established to be the most highly
26 mutable sites in the human genome¹²: 87% of previously reported *de novo* CpG
27 transitions at synonymous sites are observed in ExAC, indicating that our sample sizes
28 are beginning to approach saturation of this class of variation. This saturation is
29 detectable by a change in the discovery rate at subsets of the ExAC data set, beginning
30 at around 20,000 individuals (Figure 2b), indicating that ExAC is the first human dataset
31 large enough for this effect to be directly observed.

32

33 Mutational recurrence has a marked effect on the frequency spectrum in the ExAC data,
34 resulting in a depletion of singletons at sites with high mutation rates (Figure 2c). We

1 observe a correlation between singleton rates (the proportion of variants seen only once
2 in ExAC) and site mutability inferred from sequence context¹³ ($r = -0.98$; $p < 10^{-50}$;
3 Extended Data Figure 4d): sites with low predicted mutability have a singleton rate of
4 60%, compared to 20% for sites with the highest predicted rate (CpG transitions; Figure
5 2C). Conversely, for synonymous variants, CpG variants are approximately twice as
6 likely to rise to intermediate frequencies: 16% of CpG variants are found in at least 20
7 copies in ExAC, compared to 8% of transversions and non-CpG transitions, suggesting
8 that synonymous CpG transitions have on average two independent mutational origins in
9 the ExAC sample. Recurrence at highly mutable sites can further be observed by
10 examining the population sharing of doubleton synonymous variants (variants occurring
11 in only two individuals in ExAC). For low-mutability mutations (especially transversions),
12 these variants are more likely to be observed in a single population (representing a
13 single mutational origin), while CpG transitions are more likely to be found in two
14 separate populations (representing independent mutational events); as such, site
15 mutability and probability of observation in two populations is significantly correlated ($r =$
16 0.884; Figure 2d).

17
18 We also explored the prevalence and functional impact of multinucleotide
19 polymorphisms (MNPs), clusters of base substitutions on the same haplotype. We used
20 a read-based phasing pipeline to assess all sites where multiple substitutions were
21 observed within the same codon in at least one individual. We found 5,945 MNPs in
22 ExAC (Extended Data Figure 3a), with an average of 23 per sample, where analysis of
23 the underlying SNPs without correct haplotype phasing would result in altered
24 interpretation. These include 647 instances where the effect of a protein-truncating
25 variant (PTV) variant is eliminated by an adjacent SNP (rescued PTV) and 131 instances
26 where underlying synonymous or missense variants result in PTV MNPs (gained PTV).
27 We identified rescued PTV MNPs in *MLH1* (where PTV variants are associated with
28 autosomal dominant Lynch syndrome), and *FANCA* (autosomal recessive Fanconi
29 anemia)^{14,15}. We also identified an ExAC sample as a carrier for a gained PTV MNP in
30 *MUTYH*, where homozygous PTV variants are known to cause *MUTYH*-associated
31 polyposis¹⁶. Additionally our analysis revealed 10 MNPs that have previously been
32 reported as disease causing mutations in HGMD (Supplementary Information Table 9),
33 including a stop-gained MNP in *COH1* which has previously been identified as a

1 recessive cause of Cohen syndrome¹⁷. We note that these variants would be missed by
2 virtually all currently available variant calling and annotation pipelines.

3

4 **Inferring variant deleteriousness and gene constraint**

5 Deleterious variants are expected to have lower allele frequencies than neutral ones,
6 due to negative selection. This theoretical property has been demonstrated previously in
7 human population sequencing data^{18,19} and here (Figure 1d, Figure 1e). This allows
8 inference of the degree of natural selection against specific functional classes of
9 variation: however, mutational recurrence as described above indicates that allele
10 frequencies observed in ExAC-scale samples are also skewed by mutation rate, with
11 more mutable sites less likely to be singletons (Figure 2c and Extended Data Figure 4d).
12 Mutation rate is in turn non-uniformly distributed across functional classes - for instance,
13 stop lost mutations can never occur at CpG dinucleotides (Extended Data Figure 4e).
14 We corrected for mutation rates (Supplementary Information) by creating a mutability-
15 adjusted proportion singleton (MAPS) metric. This metric reflects (as expected) strong
16 selection against predicted PTVs, as well as missense variants predicted by
17 conservation-based methods to be deleterious (Figure 2e).

18

19 The deep ascertainment of rare variation in ExAC also allows us to infer the extent of
20 selection against variant categories on a per-gene basis by examining the proportion of
21 variation that is missing compared to expectations under random mutation. Conceptually
22 similar approaches have been applied to smaller exome datasets^{13,20} but have been
23 underpowered, particularly for the analysis of depletion of PTVs. We compared the
24 observed number of rare (MAF <0.1%) variants per gene to an expected number derived
25 from a selection neutral, sequence-context based mutational model¹³. The model
26 performs extremely well in predicting the number of synonymous variants, which should
27 be under minimal purifying selection, per gene ($r = 0.98$; Extended Data Figure 5).

28

29 We quantified deviation from expectation with a Z score¹³, which for synonymous
30 variants is centered at zero, but has distributions that are significantly shifted towards
31 higher values (greater constraint) for both missense and PTVs (Wilcoxon $p < 10^{-50}$ for
32 both). To reduce confounding by coding sequence length for PTVs, we also developed
33 an expectation-maximization algorithm (Supplementary Information Section 4.4) using
34 the observed and expected PTV counts within each gene to separate genes into three

1 general categories: null (observed \approx expected), recessive (observed $\leq 50\%$ of expected),
2 and haploinsufficient (observed $< 10\%$ of expected). This metric – the probability of being
3 loss-of-function (LoF) intolerant (pLI) – separates genes of sufficient length into LoF
4 intolerant (pLI ≥ 0.9 , n=3,230) or LoF tolerant (pLI ≤ 0.1 , n=10,374) categories. pLI is less
5 correlated with coding sequence length ($r = 0.17$ as compared to 0.57 for the PTV Z
6 score), outperforms the PTV Z score as an intolerance metric (Supplementary
7 Information Table 11), and reveals the expected contrast between gene lists (Figure 3b).
8 pLI is also positively correlated with a gene product's number of physical interaction
9 partners ($p < 10^{-41}$). The most constrained pathways (highest median pLI for the genes in
10 the pathway) are core biological processes (spliceosome, ribosome, and proteasome
11 components; KS test $p < 10^{-6}$ for all) while olfactory receptors are among the least
12 constrained pathways (KS test $p < 10^{-16}$), demonstrated in Figure 3b and consistent with
13 previous work^{5,21–23}.

14

15 Critically, we note that LoF-intolerant genes include virtually all known severe
16 haploinsufficient human disease genes (Figure 3b), but that 79% of LoF-intolerant genes
17 have not yet been assigned a human disease phenotype despite the clear evidence for
18 extreme selective constraint (Supplementary Information 4.11). These likely represent
19 either undiscovered severe dominant disease genes, or genes in which loss of a single
20 copy results in embryonic lethality.

21

22 The most highly constrained missense (top 25% missense Z scores) and PTV (pLI ≥ 0.9)
23 genes show higher expression levels and broader tissue expression than the least
24 constrained genes²⁴ (Figure 3c). These most highly constrained genes are also depleted
25 for eQTLs ($p < 10^{-9}$ for missense and PTV; Figure 3d), yet are enriched within genome-
26 wide significant trait-associated loci ($\chi^2 p < 10^{-14}$, Figure 3e). Intuitively, genes intolerant
27 of PTV variation are dosage sensitive: natural selection does not tolerate a 50% deficit in
28 expression due to the loss of single allele. It is therefore unsurprising that these genes
29 are also depleted of common genetic variants that have a large enough effect on
30 expression to be detected as eQTLs with current limited sample sizes. However, smaller
31 changes in the expression of these genes, through weaker eQTLs or functional variants,
32 are more likely to contribute to medically relevant phenotypes. Therefore, highly
33 constrained genes are dosage-sensitive, expressed more broadly across tissues (as
34 expected for core cellular processes), and are enriched for medically relevant variation.

1

2 Finally, we investigated how these constraint metrics would stratify mutational classes
3 according to their frequency spectrum, corrected for mutability as in the previous section
4 (Figure 3f). The effect was most dramatic when considering stop-gained variants in the
5 LoF-intolerant set of genes. For missense variants, the missense Z score offers
6 information additional to Polyphen2 and CADD classifications, indicating that gene-level
7 measures of constraint offer additional information to variant-level metrics in assessing
8 potential pathogenicity.

9

10 **ExAC improves variant interpretation in Mendelian disease**

11 We assessed the value of ExAC as a reference dataset for clinical sequencing
12 approaches, which typically prioritize or filter potentially deleterious variants based on
13 functional consequence and allele frequency⁶. To simulate a Mendelian variant analysis,
14 we filtered variants in 100 ExAC exomes per continental population against ESP (the
15 previous default reference data set for clinical analysis) or the remainder of ExAC,
16 removing variants present at $\geq 0.1\%$ allele frequency, a filter recommended for dominant
17 disease variant discovery⁶. Filtering on ExAC reduced the number of candidate protein-
18 altering variants by 7-fold compared to ESP, and was most powerful when the highest
19 allele frequency in any one population (“popmax”) was used rather than average
20 (“global”) allele frequency (Figure 4a). ESP is not well-powered to filter at 0.1% AF
21 without removing many genuinely rare variants, as AF estimates based on low allele
22 counts are both upward-biased and imprecise (Figure 4b). We thus expect that ExAC
23 will provide a very substantial boost in the power and accuracy of variant filtering in
24 Mendelian disease projects.

25

26 Previous large-scale sequencing studies have repeatedly shown that some purported
27 Mendelian disease-causing genetic variants are implausibly common in the population^{25–}
28 ²⁷. The average ExAC participant harbors ~53 variants reported as disease-causing in
29 two widely-used databases of disease-causing variants (Supplementary Information
30 Section 5.2). Most (~41) of these are high-quality genotypes but with implausibly high
31 (>1%) AF in at least one population. We therefore hypothesized that most of the
32 supposed burden of Mendelian disease alleles per person is due not to genotyping error,
33 but rather to misclassification in databases.

34

1 We manually curated the evidence of pathogenicity for 192 previously reported
2 pathogenic variants with allele frequency >1% either globally or in South Asian or Latino
3 individuals, populations that are underrepresented in previous reference databases.
4 Nine variants had sufficient data to support disease association, typically with either mild
5 or incompletely penetrant disease effects; the remainder either had insufficient evidence
6 for pathogenicity, no claim of pathogenicity, or were benign traits (Supplementary
7 Information Section 5.3). 163 were reclassified as benign or likely benign following
8 American College of Medical Genetics guidelines²⁸. Supporting functional data were
9 reported for 18 of these variants, highlighting the need to review cautiously even variants
10 with experimental support.

11

12 We also sought phenotypic data for a subset of ExAC participants homozygous for
13 reported severe recessive disease variants, again enabling reclassification of some
14 variants as benign. North American Indian Childhood Cirrhosis is a recessive disease of
15 cirrhotic liver failure during childhood requiring liver transplant for survival to adulthood,
16 previously reported to be caused by *CIRH1A* p.R565W²⁹. ExAC contains 222
17 heterozygous and 4 homozygous Latin American individuals, with a population allele
18 frequency of 1.92%. The 4 homozygotes had no history of liver disease according to
19 available phenotype data, and recontact with additional phenotyping in two individuals
20 revealed normal liver function (Supplementary Information Table 15). Thus, despite the
21 rigorous linkage and Sanger sequencing efforts that led to the original report of
22 pathogenicity, the ExAC data demonstrate that this variant is not a fully penetrant cause
23 of severe disease, a reminder of the importance of well-matched reference populations.

24

25 The above curation efforts confirm the importance of allele frequency filtering in analysis
26 of candidate disease variants. However, literature and database errors are prevalent
27 even at lower allele frequencies: the average ExAC exome contains 0.89 reportedly
28 Mendelian variants in well-characterized dominant disease genes³⁰ at <1% popmax AF
29 and 0.20 at <0.1% popmax AF. This inflation likely results from a combination of false
30 reports of pathogenicity and incomplete penetrance, as we show for *PRNP* in the
31 accompanying work [Minikel et al, submitted]. The abundance of rare functional variation
32 in many disease genes in ExAC is a reminder that such variants should not be assumed
33 to be causal or highly penetrant without careful segregation or case-control analysis^{28,7}.

34

1 **Impact of rare protein-truncating variants**

2 We investigated the distribution of PTVs, variants predicted to disrupt protein-coding
3 genes through the introduction of a stop codon or frameshift or the disruption of an
4 essential splice site; such variants are expected to be enriched for complete loss-of-
5 function of the impacted genes. Naturally-occurring PTVs in humans provide a model for
6 the functional impact of gene inactivation, and have been used to identify many genes in
7 which LoF causes severe disease³¹, as well as rare cases where LoF is protective
8 against disease³².

9

10 Among the 7,404,909 HQ variants in ExAC, we found 179,774 high-confidence PTVs (as
11 defined in Supplementary Information Section 6), 121,309 of which are singletons. This
12 corresponds to an average of 85 heterozygous and 35 homozygous PTVs per individual
13 (Figure 5a). The diverse nature of the cohort enables the discovery of substantial
14 numbers of novel PTVs: out of 58,435 PTVs with an allele count greater than one,
15 33,625 occur in only one population. However, while PTVs as a category are extremely
16 rare, the majority of the PTVs found in any one person are common, and each individual
17 has only ~2 singleton PTVs, of which 0.14 are found in PTV-constrained genes (pLI
18 >0.9). The site frequency spectrum of these variants across the populations represented
19 in ExAC recapitulates known aspects of demographic models, including an increase in
20 intermediate-frequency (1%-5%) PTVs in Finland³³ and relatively common ($>0.1\%$) PTVs
21 in Africans (Figure 5b).

22

23 Using a sub-sampling approach, we show that the discovery of both heterozygous
24 (Figure 5c) and homozygous (Figure 5d) PTVs scales very differently across human
25 populations, with implications for the design of large-scale sequencing studies for the
26 ascertainment of human “knockouts” described below.

27

28 **Discussion**

29 Here we describe the generation and analysis of the most comprehensive catalogue of
30 human protein-coding genetic variation to date, incorporating high-quality exome
31 sequencing data from 60,706 individuals of diverse geographic ancestry. The resulting
32 call set provides unprecedented resolution for the analysis of very low-frequency protein-
33 coding variants in human populations, as well as a powerful resource for the clinical
34 interpretation of genetic variants observed in disease patients. The complete frequency

1 and annotation data from this call-set has been made freely available through a public
2 website [exac.broadinstitute.org].

3

4 The very large sample size of ExAC also provides opportunities for a high-resolution
5 analysis of the sensitivity of human genes to functional variation. While previous sample
6 sizes have been adequately powered for the assessment of gene-level intolerance to
7 missense variation^{13,20}, ExAC provides for the first time sufficient power to investigate
8 genic intolerance to PTVs, highlighting 2,557 LoF-intolerant genes for which human
9 disease phenotypes have not yet been identified. These unassociated genes are likely to
10 fall into two main categories: those that cause severe haploinsufficient disease that has
11 not yet been genetically characterized, and those where heterozygous inactivation
12 results in embryonic lethality. In the accompanying work [Ruderfer et al., to be co-
13 submitted] we show that ExAC similarly provides power to identify genes intolerant of
14 copy number variation. Quantification of genic intolerance to both classes of variation will
15 provided added power to disease studies.

16

17 The ExAC resource provides the largest database to date for the estimation of allele
18 frequency for protein-coding genetic variants, providing a powerful filter for analysis of
19 candidate pathogenic variants in severe Mendelian diseases. Frequency data from ESP¹
20 have been widely used for this purpose, but those data are limited by population
21 diversity and by resolution at allele frequencies $\leq 0.1\%$. ExAC therefore provides
22 substantially improved power for Mendelian analyses, although it is still limited in power
23 at lower allele frequencies, emphasizing the need for more sophisticated pathogenic
24 variant filtering strategies alongside on-going data aggregation efforts. ExAC also
25 highlights an unexpected tolerance of many disease genes to functional variation, and
26 reveals that the literature and public databases contain an inflated number of reportedly
27 pathogenic variants across the frequency spectrum, indicating a need for stringent
28 criteria for assertions of pathogenicity.

29

30 Finally, we show that different populations confer different advantages in the discovery
31 of gene-disrupting PTVs, providing guidance for projects seeking to identify human
32 “knockouts” to understand gene function. Individuals of African ancestry have more
33 PTVs (140 on average), with this enrichment most pronounced at allele frequencies
34 above 1% (Figure 5b). Finnish individuals, as a result of a population bottleneck, are

1 depleted at the lowest (<0.1%) allele frequencies but have a peak in frequency at 1-5%
2 (Figure 5b). However, these differences are diminished when considering only LoF-
3 constrained (pLI > 0.9) genes (Extended Data Figure 10). Sampling multiple populations
4 would likely be a fruitful strategy for a researcher investigating common PTV variation.
5 However, discovery of homozygous PTVs is markedly enhanced in the South Asian
6 samples, which come primarily from a Pakistani cohort with 38.3% of individuals self-
7 reporting as having closely related parents, emphasizing the extreme value of
8 consanguineous cohorts for “human knockout” discovery (Figure 5d) [Saleheen *et al.*, to
9 be co-submitted].

10
11 Even with this unprecedented collection of jointly processed exomes, many limitations
12 remain. First, most ExAC individuals were ascertained for the presence or absence of a
13 biomedically important disease and thus are not a random sampling of the population.
14 To minimize this bias, we have made every effort to exclude severe pediatric diseases,
15 but nevertheless, the inclusion of both cases and controls for several polygenic disorders
16 means that ExAC may contain higher counts of disease-associated variants for certain
17 phenotypes³⁴. Second, future reference databases would benefit from including a
18 broader sampling of human diversity, particularly from the Middle East, a population not
19 represented in the present dataset. Third, some protein-coding exons lack coverage in
20 ExAC entirely or have coverage levels confounded with exome capture technology,
21 which in turn is confounded with cohort and continental population. Fourth, protein-
22 coding exons are only one source of functionally important variation, and the future
23 inclusion of whole genomes will also be critical to enable ascertainment of additional
24 classes of variation and the identification of constrained regions outside of protein-
25 coding sequence.

26
27 While the ExAC dataset dramatically exceeds the scale of previously available frequency
28 reference datasets, much remains to be gained by further increases in sample size.
29 Indeed, the fact that even the rarest transversions have mutational rates¹³ on the order
30 of 1×10^{-9} implies that almost all possible non-lethal SNVs likely exist in some person on
31 Earth. ExAC already includes >70% of all possible protein-coding CpG transitions at
32 well-covered sites; order of magnitude increases in sample size will eventually lead to
33 saturation of other classes of variation.

34

1 ExAC was made possible by the willingness of multiple large disease-focused consortia
2 to share their raw data, and by the availability of the software and computational
3 resources required to create a harmonized variant call set on the scale of tens of
4 thousands of samples. The creation of yet larger reference variant databases will require
5 continued emphasis on the value of public data sharing.

6
7

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- 12
- 13

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9 J.G.W.,M.J.D.,D.G.M. contributed to the design and conduct of the various exome
10 sequencing studies and critical review of manuscript.

11

12 **Author Information**

13 P.F.S is a scientific advisor to Pfizer.

14 ExAC data set is publicly available at <http://www.exac.broadinstitute.org>

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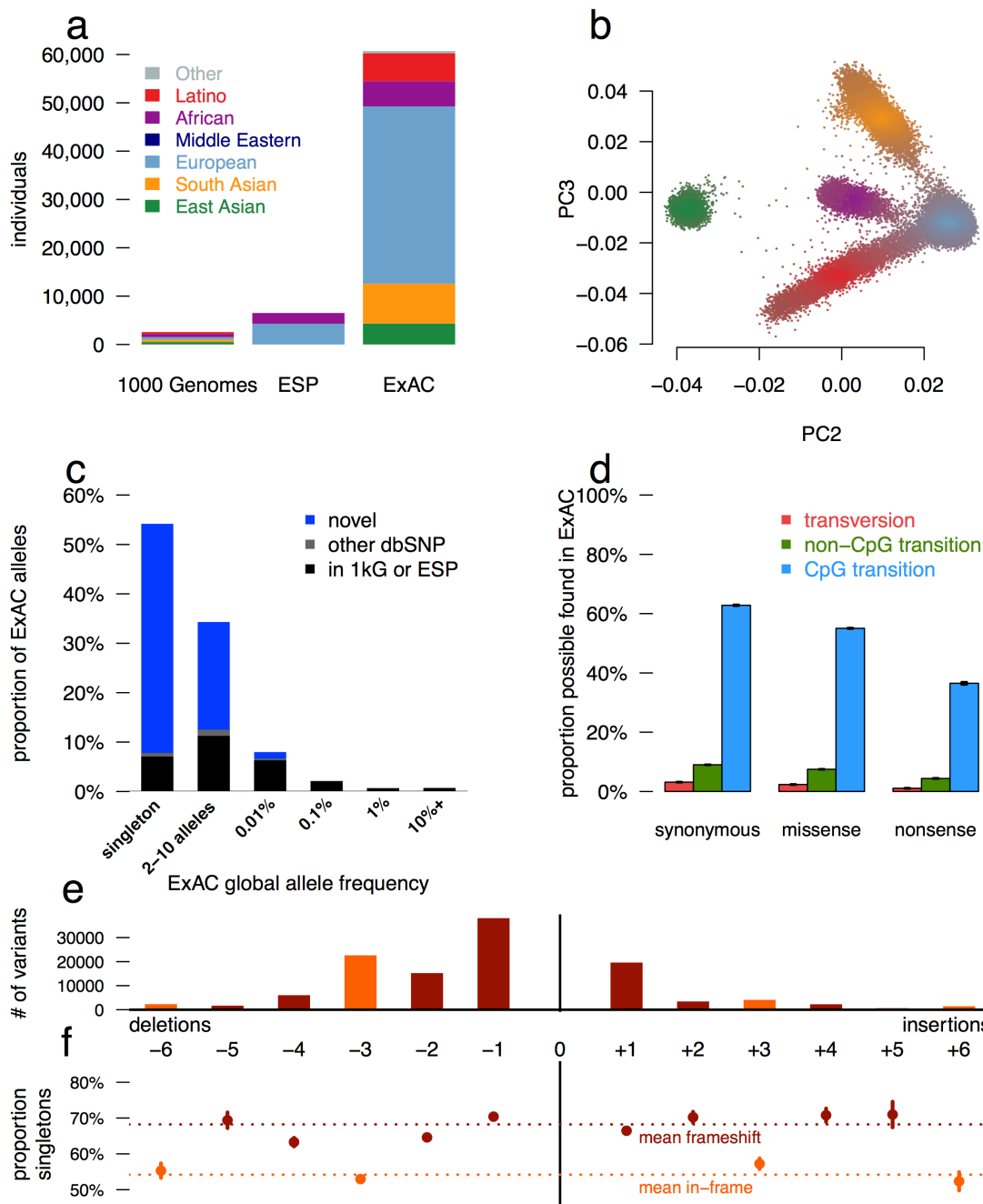
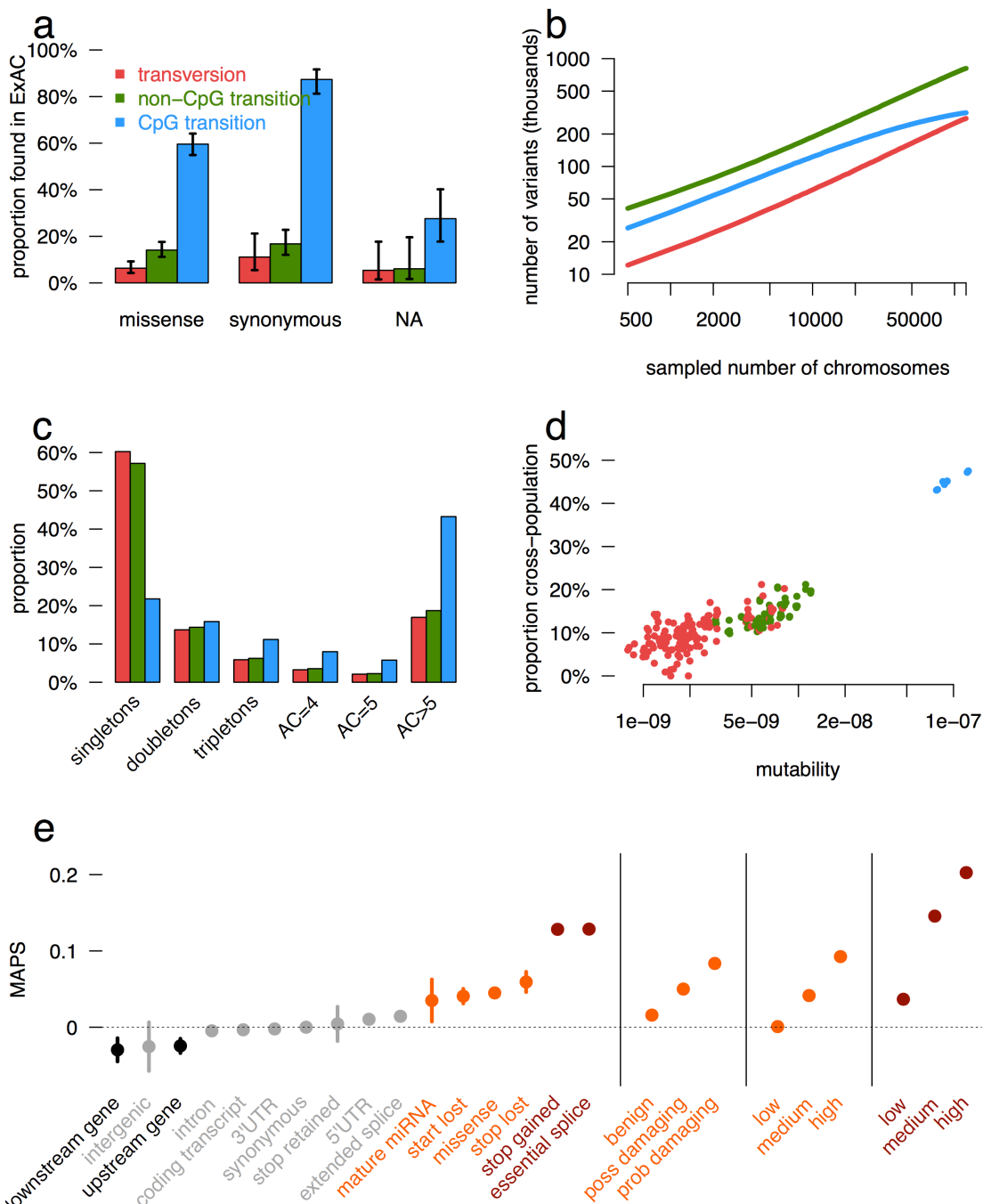


Figure 1. Patterns of genetic variation in 60,706 humans. a) The size and diversity of public reference exome datasets. ExAC exceeds previous datasets in size for all studied populations. b) Principal component analysis (PCA) dividing ExAC individuals into five continental populations. PC2 and PC3 are shown; additional PCs are in Extended Data Figure 2a. c) The allele frequency spectrum of ExAC highlights that the majority of genetic variants are rare and novel. d) The proportion of possible variation observed by mutational context and functional class. Over half of all possible CpG transitions are observed. e-f) The number (e) and frequency distribution (proportion singleton; f) of indels, by size. Compared to in-frame indels, frameshift variants are greater in number and are more common (have a lower proportion of singletons, a proxy for predicted deleteriousness on gene product).

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 2 **Figure 2. Mutational recurrence at large sample sizes.** a) Number of unique variants observed, by
 3 mutational context, as a function of number of individuals (down-sampled from ExAC). CpG transitions,
 4 the most likely mutational event, begin reaching saturation at ~20,000 individuals. B) Proportion of
 5 validated *de novo* variants from two external datasets that are independently found in ExAC, separated by
 6 functional class and mutational context. Error bars represent standard error of the mean. Colors are
 7 consistent in a-d. c) The site frequency spectrum is shown for each mutational context. d) For doubletons
 8 (variants with an allele count of 2), mutation rate is positively correlated with the likelihood of being
 9 found in two individuals of different continental populations. e) The mutability-adjusted proportion of
 10 singletons (MAPS) is shown across functional classes. Error bars represent standard error of the mean of
 11 the proportion of singletons.
 12

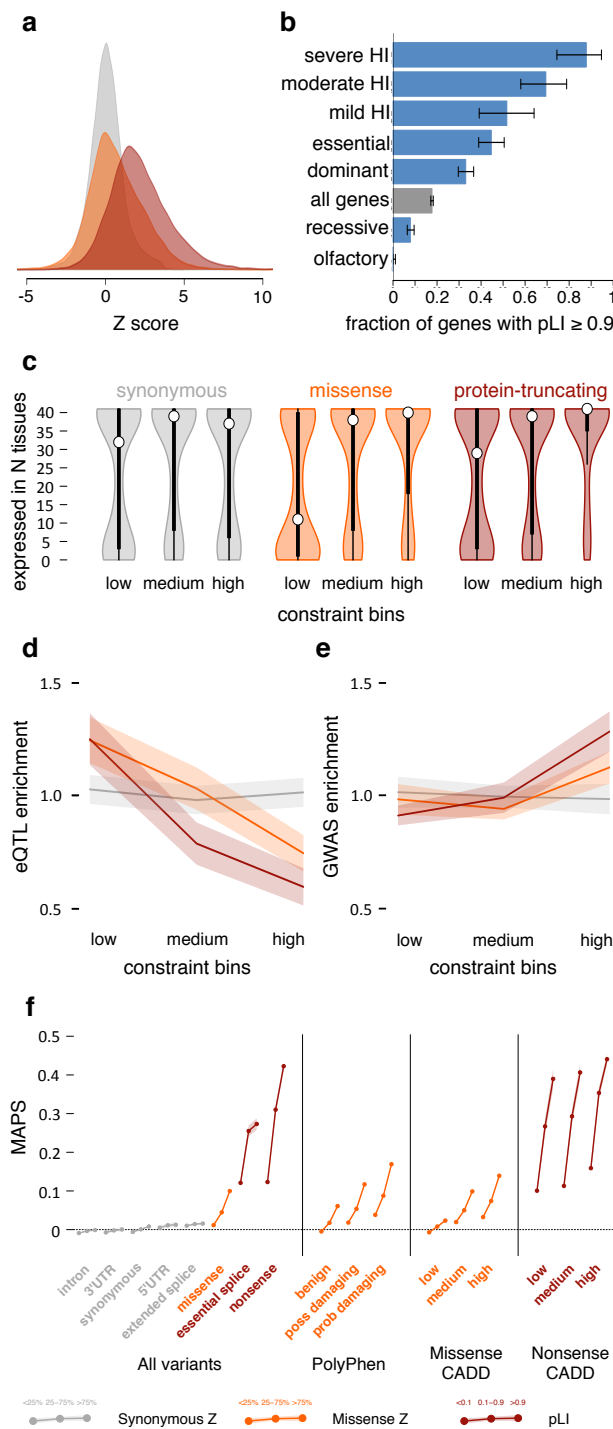
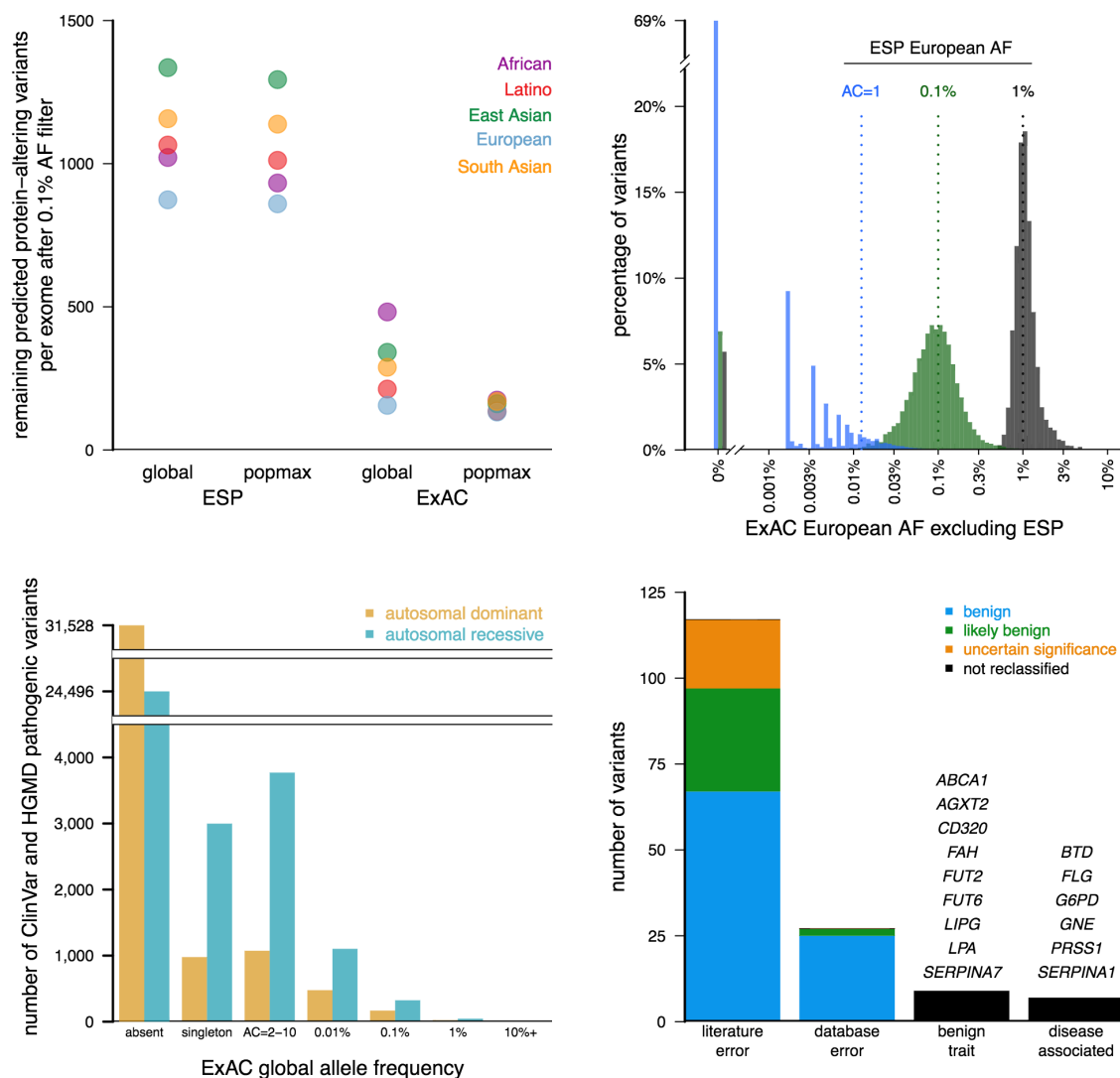
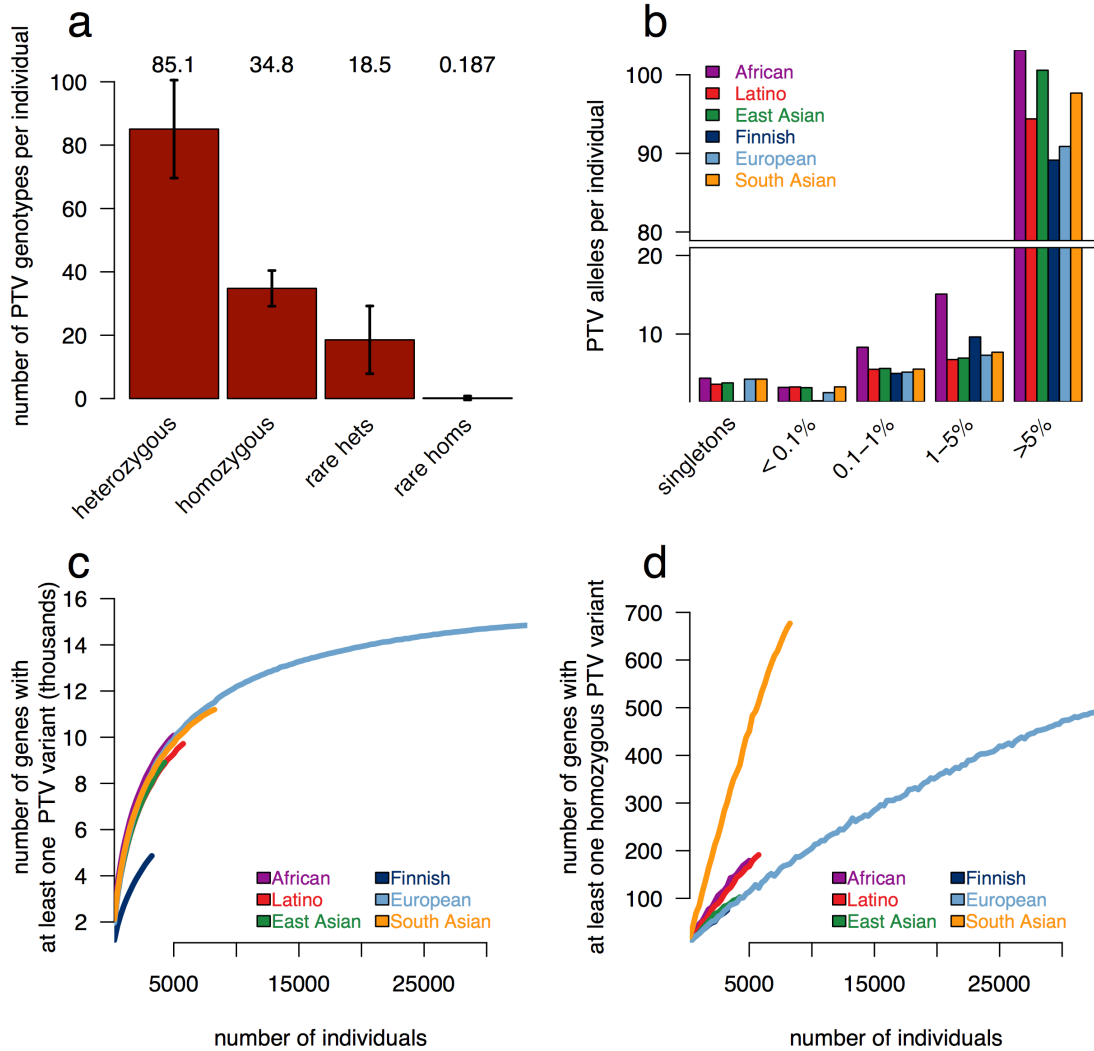


Figure 3. Quantifying intolerance to functional variation in genes and gene sets. a) Histograms of constraint Z scores [Samocha 2014] for 18,225 genes. This measure of departure of number of variants from expectation is normally distributed for synonymous variants, but right-shifted (higher constraint) for missense and protein-truncating variants (PTVs), indicating that more genes are intolerant to these classes of variation. b) The proportion of genes that are very likely intolerant of loss-of-function variation ($pLI \geq 0.9$) is highest for ClinGen haploinsufficient genes, and stratifies by the severity and age of onset of the haploinsufficient phenotype. Genes essential in cell culture and dominant disease genes are likewise enriched for intolerant genes, while recessive disease genes and olfactory receptors have fewer intolerant genes. Black error bars indicate 95% confidence intervals (CI). c) Synonymous Z scores show no correlation with the number of tissues in which a gene is expressed, but the least missense- and PTV-constrained genes tend to be expressed in fewer tissues. Thick black bars indicate the first to third quartiles, with the white circle marking the median. d) Highly missense- and PTV-constrained genes are less likely to have eQTLs discovered in GTEx as the average gene. Shaded regions around the lines indicate 95% CI. e) Highly missense- and PTV-constrained genes are more likely to be adjacent to GWAS signals than the average gene. Shaded regions around the lines indicate 95% CI. f) MAPS (Figure 2d) is shown for each functional category, broken down by constraint score bins as shown. Missense and PTV constraint score bins provide information about natural selection at least partially orthogonal to MAPS, PolyPhen, and CADD scores, indicating that this metric should be useful in identifying variants associated with deleterious phenotypes. Shaded regions around the lines indicate 95% CI. For panels a,c-f: synonymous shown in gray, missense in orange, and protein-truncating in maroon.



1
2 **Figure 4. Filtering for Mendelian variant discovery.** a) Predicted missense and protein-truncating variants
3 in 500 randomly chosen ExAC individuals were filtered based on allele frequency information from ESP, or
4 from the remaining ExAC individuals. At a 0.1% allele frequency (AF) filter, ExAC provides greater power to
5 remove candidate variants, leaving an average of 154 variants for analysis, compared to 1090 after
6 filtering against ESP. Popmax AF also provides greater power than global AF, particularly when
7 populations are unequally sampled. b) Estimates of allele frequency in Europeans based on ESP are more
8 precise at higher allele frequencies. Sampling variance and ascertainment bias make AF estimates
9 unreliable, posing problems for Mendelian variant filtration. 69% of ESP European singletons are not seen
10 a second time in ExAC (tall bar at left), illustrating the dangers of filtering on very low allele counts. c)
11 Allele frequency spectrum of disease-causing variants in the Human Gene Mutation Database (HGMD)
12 and/or pathogenic or likely pathogenic variants in ClinVar for well characterized autosomal dominant and
13 autosomal recessive disease genes³⁰. Most are not found in ExAC; however, many of the pathogenic
14 variants found in ExAC are at too high a frequency to be consistent with disease prevalence and
15 penetrance. d) Literature review of variants with >1% global allele frequency or >1% Latin American and
16 South Asian population allele frequency confirmed there is insufficient evidence for pathogenicity for the
17 majority of these variants. Variants were reclassified by ACMG guidelines²⁸.



1
2 **Figure 5. Protein-truncating variation in ExAC.** a) The average ExAC individual has 85 heterozygous and 35
3 homozygous protein-truncating variants (PTVs), of which 18 and 0.19 are rare (<0.1% popmax AF),
4 respectively. Error bars represent standard deviation. b) Breakdown of PTVs per individual (a) by popmax
5 AF bin. Across all populations, most PTVs found in a given individual are common (>5% popmax AF). c-d)
6 Number of genes with at least one PTV (c) or homozygous PTV (d) as a function of number of individuals,
7 downsampld from ExAC.
8
9