Mapping information-rich genotype-phenotype landscapes with genome-scale Perturb-seq

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Abstract

A central goal of genetics is to define the relationships between genotypes and phenotypes. High-content phenotypic screens such as Perturb-seq (pooled CRISPR-based screens with single-cell RNA-sequencing readouts) enable massively parallel functional genomic mapping but, to date, have been used at limited scales. Here, we perform genome-scale Perturb-seq targeting all expressed genes with CRISPR interference (CRISPRi) across >2.5 million human cells and present a framework to power biological discovery with the resulting genotype-phenotype map. We use transcriptional phenotypes to predict the function of poorly-characterized genes, uncovering new regulators of ribosome biogenesis (including *CCDC86*, *ZNF236*, and *SPATA5L1*), transcription (*C7orf26*), and mitochondrial respiration (*TMEM242*). In addition to assigning gene function, single-cell transcriptional phenotypes allow for in-depth dissection of complex cellular phenomena – from RNA processing to differentiation. We leverage this ability to systematically identify the genetic drivers and consequences of aneuploidy and to discover an unanticipated layer of stress-specific regulation of the mitochondrial genome. Our information-rich genotype-phenotype map reveals a multidimensional portrait of gene function and cellular behavior.

52 **Main Text**

53 Mapping the relationship between genetic changes and their phenotypic consequence is
54 critical to understanding gene and cellular function. This mapping is traditionally carried out in
55 either of two ways. A phenotype-centric, "forward genetic" approach reveals the genetic changes
56 that drive a phenotype of interest. Conversely, a gene-centric, "reverse genetic" approach catalogs
57 the diverse phenotypes caused by a defined genetic change.

58 Recent technological developments have advanced both forward and reverse genetic
59 efforts (*1*). CRISPR-Cas tools now enable the deletion, mutation, repression, or activation of genes
60 with ease (*2*). In forward genetic screens, CRISPR-Cas systems can be used to generate cells with
61 diverse genetic perturbations. This pool of perturbed cells can then be subjected to a selective
62 pressure, with phenotypes assigned to genetic perturbations by sequencing. Forward genetic
63 screens provide powerful tools for the identification of cancer dependencies, essential cellular
64 machinery, differentiation factors, and suppressors of genetic diseases (*3–6*). In parallel, dramatic
65 improvements in molecular phenotyping now allow for single-cell readouts of epigenetic,
66 transcriptomic, proteomic, and imaging information (*7*). Applied to reverse genetics, single-cell
67 profiling can refine the understanding of how select genetic perturbations affect cell types and cell
68 states.

69 However, both phenotype-centric and gene-centric approaches suffer conceptual and
70 technical limitations. Pooled forward genetic screens typically use low-dimensional phenotypes
71 (e.g., growth, marker gene expression, drug resistance) for selection. The use of simple phenotypes
72 can conflate genes acting via different mechanisms, requiring extensive follow-up studies to
73 disentangle genetic pathways (*8*). Additionally, in forward genetics, serendipitous discovery is
74 constrained by the prerequisite of selecting phenotypes prior to screening. On the other hand, while
75 reverse genetic approaches enable the study of multidimensional and complex phenotypes, they
76 have typically been restricted in scale to rationally chosen targets, limiting the ability to make
77 systematic comparisons.

78 Single-cell CRISPR screens present a solution to these problems. These screens
79 simultaneously read out the genetic perturbation and high-dimensional phenotype of individual
80 cells in a pooled screening format, thus combining the throughput of forward genetic screens with
81 the rich phenotypes of reverse genetics. While these approaches initially focused on transcriptomic
82 phenotypes (e.g., Perturb-seq, CROP-seq) (*9–13*), technical advances have enabled their
83 application to epigenetic (*14*), imaging (*15*), or multimodal phenotypes (*16–18*). From these rich

data, it is possible to identify genetic perturbations that cause a specific behavior as well as to catalog the spectrum of phenotypes associated with each genetic perturbation. Despite the promise of single-cell CRISPR screens, their use has generally been limited to studying at most a few hundred genetic perturbations, typically chosen with a bias towards predefined biological questions.

We reasoned that there would be unique value to genome-scale single-cell CRISPR screens. For example, while the number of perturbations scales linearly with experimental cost, the number of pairwise comparisons in a screen—and thus its utility for unsupervised classification of gene function—scales quadratically. Similarly, in large-scale screens, the diversity of perturbations allows one to explore the range of cell states that can be revealed by rich phenotypes. Additionally, as many human genes are well-characterized, these genes serve as natural controls to anchor the interpretation of observations in comprehensive datasets. Finally, genome-scale experiments could help address fundamental biological questions, such as what fraction of genetic changes elicit global transcriptional phenotypes and how transcriptional programs are rewired between cell types, with implications for understanding the organizing principles of cellular systems (*19*).

Here we perform the first genome-scale Perturb-seq screens. We use a compact, multiplexed CRISPR interference (CRISPRi) library to assay thousands of loss-of-function genetic perturbations with single-cell RNA-sequencing (scRNA-seq) in chronic myeloid leukemia (K562) and retinal pigment epithelial (RPE1) cell lines. Leveraging the scale and diversity of these perturbations across >2.5 million cells, we show that Perturb-seq can be used to study numerous complex cellular phenotypes—from RNA splicing to differentiation to chromosomal instability—in a single screen. We demonstrate how the interpretability of scRNA-seq phenotypes enables the discovery of gene function and extensively validate our findings with orthogonal experiments. Finally, we invert our analysis to focus on regulatory networks rather than genetic perturbations and uncover unanticipated stress-specific regulation of the mitochondrial genome. In sum, we use Perturb-seq to reveal a multidimensional portrait of cellular behavior, gene function, and regulatory networks that advances the goal of creating comprehensive genotype-phenotype maps.

116 **Results**

117

118 ***A multiplexed CRISPRi strategy for genome-scale Perturb-seq***

119

120 Perturb-seq uses scRNA-seq to concurrently read out the CRISPR single-guide RNAs
121 (sgRNA) (i.e., genetic perturbation) and transcriptome (i.e., high-dimensional phenotype) of single
122 cells in a pooled format (Fig. 1A). To enable genome-scale Perturb-seq, we considered key
123 parameters that would increase scalability and data quality, such as the genetic perturbation
124 modality and sgRNA library.

125 Although Perturb-seq is compatible with a range of CRISPR-based perturbations including
126 knockout (*10–12*), knockdown (CRISPRi) (*9*), or activation (CRISPRa) (*20*), we elected to use
127 CRISPRi for several reasons. First, CRISPRi allows the efficacy of the genetic perturbation,
128 knockdown, to be directly measured from scRNA-seq. Exploiting this feature allowed us to target
129 each gene in our library with a single element and empirically exclude unperturbed genes from
130 downstream analysis. Second, CRISPRi tends to yield more homogeneous genetic perturbation
131 than nuclease-based CRISPR knockout, which can generate a subset of cells bearing active in-
132 frame indels (*21*). The relative homogeneity of CRISPRi limits selection for unperturbed cells,
133 especially when studying essential genes. Third, unlike nuclease-based gene knockout, CRISPRi
134 does not lead to activation of the DNA damage response which can alter cell state and
135 transcriptional signatures (*22*).

136 To improve scalability, we optimized our CRISPRi sgRNA libraries. To maximize
137 CRISPRi efficacy, we used multiplexed CRISPRi libraries in which each construct contains two
138 distinct sgRNAs targeting the same gene (table S1-S3; see *Methods*) (*13*). To avoid low
139 representation of sgRNAs targeting essential genes, we performed preliminary growth screens and,
140 during library synthesis, overrepresented constructs that caused strong growth defects (fig. S1A-
141 D).

142 Next, we devised a three-pronged Perturb-seq screening approach encompassing multiple
143 timepoints and cell types (Fig. 1A). As a primary cell line, we studied chronic myeloid leukemia
144 (CML) K562 cells engineered to express the CRISPRi effector protein dCas9-KRAB (*23*). In this
145 cell line, we performed two Perturb-seq screens: one targeting all expressed genes sampled at day
146 8 after transduction (n=9,866 genes; n=10,673 total perturbations; some genes have multiple
147 independent transcripts) and another targeting common essential genes, which was sampled at day

148 6 after transduction (n=2,057 genes; n=2,176 total perturbations). As a secondary cell line, we used
149 RPE1 cells engineered to express dCas9 fused to a KRAB domain derived from the gene *ZIM3*,
150 which was recently shown to yield improved transcriptional repression compared to the *KOX1*-
151 derived KRAB domain used in previous CRISPRi experiments (*24*). In contrast to K562 cells,
152 RPE1 cells are a non-cancerous retinal pigment epithelial cell line that are hTERT-immortalized,
153 near-euploid, adherent, and p53-positive. In RPE1 cells, we performed a screen targeting common
154 essential genes plus a subset of nonessential genes that produced phenotypes in K562 cells sampled
155 at day 7 after transduction (n=2,393 genes; n=2,549 total perturbations).
156 We conducted these three screens with 10x Genomics droplet-based 3' scRNA-seq and
157 direct sgRNA capture (*13*). After sequencing and read alignment, we performed sgRNA
158 identification and removed any cells bearing sgRNAs targeting different genes, which are an
159 expected byproduct of lentiviral recombination between sgRNA cassette or doublet encapsulation
160 during scRNA-seq. In total, we obtained >2.5 million high-quality cells with a median coverage
161 of >100 cells per perturbation (fig. S1 E-G; table S4-S6). We observed a median target knockdown
162 of 85.5% in K562 cells and 91.6% in RPE1 cells (Fig. 1B), confirming both the efficacy of our
163 CRISPRi libraries and the fidelity of sgRNA assignment (*13*). The difference in performance
164 between these cell lines was likely due to the use of the optimized *ZIM3*-derived KRAB domain
165 in the RPE1 cells, suggesting that future efforts would benefit from improved CRISPRi efficacy.
166
167 ***A robust computational framework to detect transcriptional phenotypes***
168
169 The scale of our experiment provided a unique opportunity to ask what fraction of genetic
170 perturbations cause a transcriptional phenotype, a preliminary requirement for inferring gene
171 function. Significant transcriptional phenotypes can take many forms, ranging from altered
172 occupancy of a given cell state to focused changes in the expression level of a small number of
173 target genes. To contend with this diversity, we created a robust framework capable of detecting
174 transcriptional changes between groups of cells in our data. Our experimental design included
175 many control cells bearing diverse non-targeting sgRNAs. These allow for internal *z*-normalization
176 of expression measurements, and we found that this procedure corrected for batch effects that
177 resulted from parallelized scRNA-seq and sequencing (fig. S2). As Perturb-seq captures single-
178 cell genetic perturbation identities in a pooled format, we can use statistical approaches that treat
179 each cell as an independent experimental sample. In general, we chose to use conservative, non-

180 parametric statistical tests to detect transcriptional changes rather than making specific
181 assumptions about the underlying distribution of gene expression levels.

182 First, we examined global transcriptional changes using a permuted energy distance test
183 (see *Methods*). We compared cells bearing each genetic perturbation to non-targeting control cells
184 at the level of principal components (approximating global transcriptional features like cell state
185 and gene expression programs). Relative to a permuted null distribution, this test asks whether
186 cells carrying a given genetic perturbation could have been drawn from the control population. By
187 this metric, we found that 2,987 of 9,608 genetic perturbations targeting a primary transcript
188 (31.1%) compared to 11 of 585 non-targeting controls (1.9%) caused a significant transcriptional
189 phenotype in K562 cells.

190 While sensitive, the energy distance test assays global shifts in expression without
191 providing insight into which specific transcripts are altered. To detect individual differentially
192 expressed genes, we applied the Anderson-Darling (AD) test to compare the distribution of
193 expression levels for each gene in cells bearing each genetic perturbation against control cells.
194 Importantly, the AD test is sensitive to transcriptional changes in a subset of cells, enabling us to
195 find differences even when phenotypes have incomplete penetrance. With the AD test, we found
196 2,935 of 9,608 genetic perturbations targeting a primary transcript (30.5%) compared to 12 of 585
197 non-targeting controls (2.1%) caused >10 differentially expressed genes in K562 cells. These
198 results were well-correlated between time points and cell types (fig. S3A,B; tables S4-S6) and
199 concordant with the energy distance test (78.7% concordance by Jaccard index).

200 We then explored features of genetic perturbations that predict a transcriptional phenotype.
201 We found that the strength of the transcriptional response was correlated with the strength of the
202 growth defect (Spearman's rho = –0.51) with 86.6% of essential genetic perturbations (gamma <
203 –0.1) leading to a significant transcriptional response in K562 cells (Fig. 1C; fig. S3C,D). A
204 substantial number of genetic perturbations that cause a transcriptional phenotype nonetheless
205 have a negligible growth phenotype (n=771; fig. S3E), indicating that many genetic perturbations
206 influence cell state but not growth or survival. We also found that highly expressed genes were
207 more likely to produce transcriptional phenotypes (Spearman's rho = 0.42) (fig. S3C).

208 Considering that some of our genetic perturbations did not yield strong on-target
209 knockdown, our estimate of the fraction of genetic perturbations that cause a transcriptional
210 phenotype is likely to be a lower bound. While a fraction of phenotypes may result from off-target
211 effects, an advantage of Perturb-seq is the ability to directly detect potential off-target activities

212 such as the knockdown of neighboring genes. Consistent with earlier studies (*25*), we found that
213 ~7.5% of perturbations caused significant knockdown of a neighboring gene in K562 cells, but
214 neighbor gene knockdown was not enriched in genetic perturbations with a negligible growth
215 defect that produced a transcriptional phenotype (fig. S4). Taken together, these results present a
216 coherent picture where knockdown of a significant fraction of expressed genes causes a
217 transcriptional response.
218
219 ***Annotating gene function from transcriptional phenotypes***
220
221 Previous Perturb-seq screens have focused on targeted sets of genetic perturbations that are
222 often related biologically, such as genes identified in forward genetic screens. Our large-scale
223 screen targeting all expressed genes in K562 cells presented a unique opportunity to assess how
224 well transcriptional phenotypes can resolve gene function when used in an unbiased manner.
225 We focused on a subset of 1,973 perturbations that had strong transcriptional phenotypes
226 (>50 differentially expressed genes by AD test) (Fig. 2A). Because related perturbations could
227 have different magnitudes of effect, we used the correlation between mean expression profiles as
228 a scale-invariant metric of similarity.
229 To assess the extent to which correlated mean expression profiles between genetic
230 perturbations indicated common function, we compared our results to two curated sources of
231 biological relationships. First, among the 1,973 targeted genes, there were 327 protein complexes
232 from the CORUM 3.0 database with at least two thirds of the complex members present,
233 representing 14,165 confirmed protein-protein interactions (*26*). The corresponding expression
234 profile correlations were markedly stronger (median correlation 0.61) than the background
235 distribution of all possible gene pairs (median correlation 0.10) (Fig. 2B). Second, we compared
236 the correlation between genetic perturbations to the STRING database of known and predicted
237 protein-protein interactions, which had scores for 243,558 of the possible gene-gene relationships
238 within our dataset (*27*). High STRING scores, reflecting high-confidence interacting proteins,
239 were also strongly associated with high expression correlations (Fig. 2C).
240 We next performed an unbiased search for global structure to group similar perturbations
241 within the dataset. We identified 64 discrete clusters based on strong intra-cluster correlations and
242 annotated their function using CORUM, STRING, and manual searches. To visualize the dataset,

243 we constructed a minimum distortion embedding that places genes with correlated expression
244 profiles close to each other in the plane and labeled the location of gene clusters (Fig. 2D).
245 Both the clusters and the embedding showed clear organization by biological function
246 spanning a diverse array of different processes including: chromatin modification; transcription;
247 mRNA splicing, capping, polyadenylation, and turnover; nonsense-mediated decay; translation;
248 post-translational modification, trafficking, and degradation of proteins; central metabolism;
249 mitochondrial transcription and translation; DNA replication; cell division; microRNA biogenesis;
250 and major signaling pathways active in K562 cells such as BCR-ABL and mTOR (table S7). We
251 further annotated the embedding visualization by labeling CORUM complexes and STRING
252 clusters whose members were placed in nearby positions, revealing structure at finer resolution
253 such as identifying the SMN complex, exon junction complex, U6 snRNP, and methylosome
254 within the spliceosome and the association of ribosome biogenesis factors with the 40S ribosomal
255 subunit.
256 In our dataset, we identified many poorly annotated genes whose perturbation led to similar
257 transcriptional responses to genes of known function, naturally predicting a role for the
258 uncharacterized genes. To orthogonally test a subset of these predictions, we selected ten poorly
259 annotated genes whose perturbation response correlated (r>0.6) with subunits and biogenesis
260 factors of either the large or small subunit of the cytosolic ribosome, which formed distinct clusters
261 in our data (fig. S5A). This included genes that had no previous association with ribosome
262 biogenesis (*CCDC86*, *CINP*, *SPATA5L1*, *ZNF236*, *C1orf131*) as well as genes that had not been
263 associated with functional defects in a particular subunit (*SPOUT1*, *TMA16*, *NOPCHAP1*, *ABCF1*,
264 and *NEPRO*). We used CRISPRi to target these genes in K562 cells and looked for evidence of
265 ribosome biogenesis defects by assessing the ratio of 28S to 18S rRNA by Bioanalyzer
266 electrophoresis. Knockdown of nine of the ten candidate factors led to substantial defects in
267 ribosome biogenesis, with the exception of *ABCF1* (Fig. 2E). In every case, the affected ribosomal
268 subunit corresponded to the Perturb-seq clustering across two independent sgRNAs. While this
269 study was in progress, another group used cryo-EM to identify C1orf131 as a core structural
270 component of the pre-A1 small subunit processome, complementing our functional evidence (*28*).
271 This validation suggests that many poorly characterized genes can be assigned functional roles
272 through Perturb-seq, although a subset of these relationships might be explained by indirect or off-
273 target effects (fig. S5B,C).

274 In total, these results show that transcriptional phenotypes revealed by Perturb-seq have
275 utility beyond studying gene regulation or transcriptional programs, and can serve as valuable tools
276 for resolving and interrogating many central processes in cell biology.
277
278 ***Delineating functional modules of the Integrator complex***
279
280 In general, perturbations to members of known protein complexes produced similar
281 transcriptional phenotypes in our dataset. Therefore, we were surprised by the wide spectrum of
282 transcriptional responses to knockdown of subunits of Integrator, a metazoan-specific essential
283 nuclear complex with roles in small nuclear RNA (snRNA) biogenesis and transcription
284 termination at paused RNA polymerase II (Fig. 3A) (*29*). Each of the fourteen core subunits of
285 Integrator was targeted in our experiment, allowing us to systematically compare their
286 transcriptional phenotypes in K562 and RPE1 cells (Fig. 3B; fig. S6A). *INTS1*, *INTS2*, *INTS5*,
287 *INTS7*, and *INTS8* formed a tight cluster which weakly correlated with *INTS6* and *INTS12*.
288 Separately, *INTS3*, *INTS4*, *INTS9*, and *INTS11* clustered together alongside splicing regulators
289 involved in snRNP assembly and the tri-snRNP. Finally, *INTS10*, *INTS13*, and *INTS14* formed
290 another discrete cluster together with *C7orf26*, an uncharacterized gene.
291 These distinct functional modules mirror the architecture of the Integrator complex
292 observed in recent structures (*30*, *31*). The INTS1-2-5-7-8 functional module contained the
293 subunits identified as the structural shoulder and backbone of Integrator. The INTS3-4-9-11
294 functional module contained the subunits identified as the structural cleavage module (as well as
295 INTS3 which was not resolved). While INTS10, INTS13, and INTS14 were not resolved in the
296 recent cryo-EM Integrator structures, these subunits have been identified as a stable biochemical
297 subcomplex (*32*, *33*).
298 Integrator is an essential and well-studied complex, so we were intrigued by the robust
299 clustering of the uncharacterized gene *C7orf26* with Integrator subunits 10, 13, and 14. To explore
300 this, we tested whether loss of C7orf26 impacted the abundance of Integrator subunits. CRISPRi-
301 based depletion of C7orf26 destabilized INTS10 in K562 cells, confirming either a regulatory or
302 protein-level relationship (Fig. 3C). Next, we checked for a biochemical interaction between these
303 proteins. Pulldown of His-INTS10 from cell lysates recovered endogenous C7orf26 alongside
304 INTS13 and INTS14 (Fig. 3D). Additionally, overexpression of C7orf26 with INTS10, INTS13,
305 and INTS14 enabled the purification of a stable INTS10-13-14-C7orf26 complex by size-

306 exclusion chromatography (Fig. 3E; fig. S6B and fig. S7). We also detected a physical interaction
307 between the Drosophila C7orf26 orthologue and fly Integrator in S2 cells and observed co-
308 essentiality between C7orf26 and INTS10, INTS13, INTS14 in the Cancer Dependency Map,
309 suggesting that this relationship is conserved across species and cell types (fig. S8). Together, these
310 results suggest that C7orf26 is a core subunit of a novel INTS10-13-14-C7orf26 Integrator module.
311 We sought to better understand the distinct transcriptional phenotypes induced by
312 knockdown of INTS10-13-14-C7orf26 compared to the shoulder/backbone and cleavage modules.
313 Comparison of the genes differentially expressed between modules did not reveal function in an
314 obvious way (fig. S6C,D), perhaps owing to the late time point assayed in our experiment. We
315 next explored the canonical role of Integrator in snRNA biogenesis. As mature snRNAs are not
316 captured in 3' scRNAs-seq, we monitored changes in global splicing as a proxy for snRNA
317 biogenesis defects. In our Perturb-seq data, we quantified changes in splicing by comparing the
318 ratio of intronic (unspliced) to exonic (spliced) reads for each gene. Validating our approach,
319 depletion of known splicing factors as well as subunits of the cleavage and shoulder/backbone
320 modules led to gross splicing defects (Fig. 3F). By contrast, depletion of subunits of the INTS10-
321 13-14-C7orf26 module did not cause a substantial splicing defect. To directly test the effect of the
322 INTS10-13-14-C7orf26 module on snRNA biogenesis, we used PRO-seq to probe the positioning
323 of active RNA-polymerase. These data confirmed that extended knockdown of the cleavage and
324 backbone/shoulder modules, but not *INTS10*, *INTS13*, or *C7orf26*, caused a dramatic increase in
325 transcriptional readthrough past the 3' cleavage site of snRNAs (Fig. 3G). In addition, the PRO-
326 seq data confirmed that loss of the INTS10-13-14-C7orf26 module causes a transcriptional
327 phenotype distinct from other modules (fig. S6E).
328 In sum, our results show that INTS10-13-14-C7orf26 represents a functionally and
329 biochemically distinct module of the Integrator complex, and we propose that *C7orf26* be renamed
330 *INTS15* for future studies (Fig. 3H). Although Integrator has been subjected to extensive structural
331 analyses, it has been difficult to resolve the INTS10-13-14 components in relation to the rest of
332 the complex. Inclusion of C7orf26 may facilitate future structural efforts. Broadly, this example
333 highlights the utility of high-dimensional functional phenotypes for the unsupervised classification
334 of protein complex subunits into functional modules.
335
336
337

338 ***Data-driven definition of transcriptional programs***

339

340 While clustering can organize genetic perturbations into pathways or complexes, it does
341 not reveal the functional consequences of perturbations. To globally summarize the genotype-
342 phenotype relationships in our dataset, we: (i) clustered genes into expression programs based on
343 their co-regulation across perturbations; (ii) clustered perturbations with strong phenotypes based
344 on their transcriptional profiles (as described above); and (iii) computed the average activity of
345 each gene expression program within each perturbation cluster (Fig. 4A,B; fig. S9A; table S7,S8;
346 see *Methods*). This map uncovered many known gene expression programs associated with genetic
347 perturbations, including upregulation of proteasomal subunits due to proteosome dysfunction (*34*),
348 activation of NFkB signaling upon loss of ESCRT proteins (*35*), downregulation of growth-related
349 genes in response to essential genetic perturbations, and upregulation of the cholesterol
350 biosynthesis pathway in response to defects in vesicular trafficking (*36*). Beyond these large-scale
351 relationships, we could also score the effects of individual genetic perturbations on different
352 expression programs. For example, our analysis delineated the canonical branches of the cellular
353 stress response into the independently regulated Unfolded Protein Response (UPR) and Integrated
354 Stress Response (ISR) (Fig. 4C) (*9*). The ISR was highly activated by loss of mitochondrial
355 proteins, aminoacyl-tRNA synthetases, and translation initiation factors, whereas the UPR was
356 activated by loss of ER-resident chaperones and translocation machinery. Collectively, this
357 analysis establishes the ability of Perturb-seq to learn regulatory circuits by leveraging the
358 variability of responses across perturbations.

359 Interestingly, our unbiased clustering uncovered many perturbations that drove the
360 expression of markers of erythroid or myeloid differentiation, consistent with the known
361 multilineage potential of K562 cells (Fig. 4D) (*37*). The scale of our experiment allowed us to
362 comprehensively search for genes whose modulation promotes cellular differentiation, an
363 application of major interest in both developmental and cancer biology. As expected, loss of
364 central regulators of erythropoiesis (*GATA1*, *LDB1*, *LMO2*, and *KDM1A*) caused myeloid
365 differentiation, whereas knockdown of *BCR-ABL* and its downstream adaptor *GAB2* induced
366 erythroid differentiation (*38*). Surprisingly, loss of a number of common essential genes (i.e.,
367 essential across cell lines in the Cancer Dependency Map) also caused expression of either myeloid
368 (e.g., Integrator subcomplex) or erythroid (e.g., NuRD complex, DNA replication machinery)
369 markers. Next, we investigated the differentiation effect of selectively essential genes, which could

370 be promising targets for differentiation therapy, analogous to ongoing efforts for KDM1A (*39*,
371 *40*). We observed that loss of *PTPN1*, a tyrosine phosphatase selectively essential in K562 cells,
372 drove myeloid differentiation. While inhibitors of PTPN1 have been developed for use in diabetes
373 and certain cancers (*41*), to our knowledge they have not been tested as a differentiation therapy.
374 Remarkably, in targeted experiments, we found that knockdown of *PTPN1* and *KDM1A* in
375 combination caused a substantial increase in differentiation and growth defect compared to either
376 single genetic perturbation, suggesting that these targets act via different cellular mechanisms (Fig.
377 4E; fig. S9B). These results highlight the utility of rich phenotypes for understanding
378 differentiation as well as nominating promising therapeutic targets or combinations.
379
380 ***Hypothesis-driven study of composite phenotypes***
381
382 We next recognized that our scRNA-seq readout could be used to study phenotypes that
383 integrate data from across the transcriptome and, therefore, would be difficult to study in
384 traditional forward genetic screens. Examples of these "composite phenotypes" include total
385 cellular RNA content and the fraction of RNA derived from transposable elements (TE). We found
386 numerous composite phenotypes under strong genetic control, with highly reproducible effects
387 across screen replicates and cell types (Fig. 4F). In the specific case of TE regulation, two major
388 classes of perturbations increased the fraction of TE RNA by affecting broad classes of elements
389 including Alu, L1, and MIR (Fig. 4G; fig. S9C). First, loss of subunits of the exosome led to a
390 substantial increase in the fraction of TE RNA, suggesting that transcripts deriving from TEs might
391 be preferentially degraded. Second, loss of the CPSF cleavage and polyadenylation complex and
392 parts of the Integrator complex produced a similar phenotype, suggesting that many of the TE
393 RNAs observed in K562 cells may be derived from failure of normal transcription termination.
394 Turning to total RNA content (Fig. 4H), we found that loss of many essential regulators of
395 S-phase and mitosis increased the RNA content of cells. This is consistent with the observation
396 that cells tend to increase their size, and thus their RNA content, as they progress through the cell
397 cycle (fig. S9D), so perturbations that arrest cells in later cell cycle stages yield increased total
398 RNA content on average. By contrast, loss of essential transcriptional machinery, including
399 general transcription factors, the Mediator complex, and transcription elongation factors,
400 decreased total RNA content. In sum, these analyses show that genome-scale Perturb-seq enables

401 hypothesis-driven exploration of complex cellular features that are challenging to study through
402 other means.
403
404 ***Exploring genetic drivers and consequences of aneuploidy in single-cells***
405
406 As Perturb-seq is a single-cell assay, it enables the study of cell-to-cell heterogeneity in
407 response to genetic perturbations. We reasoned that systematically exploring sources of
408 heterogeneity could reveal insights into phenotypes that are missed in bulk or averaged
409 measurements.
410 To assess the penetrance of perturbation-induced phenotypes, we first applied SVD-based
411 leverage scores as a metric of single-cell phenotypic magnitude (see *Methods*). In this formulation,
412 leverage scores quantify how outlying each perturbed cell's transcriptome is relative to non-
413 targeting control cells without assuming that perturbations drive a single axis of variation.
414 Supporting this approach, we found that mean leverage scores for each genetic perturbation were
415 correlated with the number of differentially expressed genes (fig. S10A, Spearman's rho = 0.71),
416 and reproducible across the day 6 and day 8 K562 experiments (fig. S10B, Spearman's rho = 0.79).
417 To quantify the degree of heterogeneity in response to genetic perturbations, we then scored
418 perturbations by the variation in single-cell leverage scores (Fig. 5A; see *Methods*). Comparing
419 leverage scores across subunits of large essential complexes, we observed evidence for both
420 biological (e.g., subcomplex function or dosage imbalance) and technical (e.g., selection to escape
421 toxic perturbations) sources of phenotypic variation in response to genetic perturbations (fig.
422 S10C-F).
423 Intriguingly, many genes implicated in chromosome segregation were among the top
424 drivers of heterogeneity, including *TTK*, *SPC25*, and *DSN1* (Fig. 5B) (*42*). We hypothesized that
425 the extreme transcriptional variability caused by these genetic perturbations might result from
426 acute changes in the copy number of individual chromosomes due to mitotic mis-segregation. To
427 explore this, we used inferCNV (*43*) to estimate single-cell DNA copy number along the genome
428 by quantifying the change in moving average gene expression compared to control cells.
429 Consistent with our hypothesis, knockdown of *TTK*, a core component of the spindle assembly
430 checkpoint (*44*), led to dramatic changes in estimated DNA copy number in both intrinsically
431 aneuploid K562 and near euploid RPE1 cells (Fig. 5C; fig. S11A). Specifically, in RPE1 cells, we
432 found that 61/80 (76%) of *TTK* knockdown cells had evidence of karyotypic changes compared to

433 274/13140 (2%) of unperturbed cells. Notably, *TTK* knockdown cells bore highly variable
434 karyotypes due to the stochastic gain or loss of chromosomes, accounting for the phenotypic
435 heterogeneity observed in these cells (Fig. 5C).

436 An important advantage of the rich data provided by Perturb-seq is the ability to dissect
437 not only perturbation-phenotype associations but also relationships between cellular phenotypes.
438 We were curious how chromosomal instability (CIN) would affect cell cycle progression in
439 euploid, p53-positive RPE1 cells versus constitutively aneuploid, p53-deficient K562 cells.
440 Expanding our analysis to all cells in our experiment independent of genetic perturbation, we found
441 that RPE1 cells with abnormal karyotypes tended to arrest in G1 or G0 of the cell cycle (G1 or G0
442 fraction 0.68 for abnormal karyotype vs. 0.44 for stable karyotype), while K562 cells with altered
443 karyotypes had less significant shifts in cell cycle occupancy (Fig. 5D,E). Within the population
444 of RPE1 cells bearing a chromosomal loss, the likelihood of cell cycle arrest directly depended on
445 the magnitude of karyotypic abnormality (fig. S11B). Additionally, we observed that cells with
446 the most severe karyotypic changes—those bearing both chromosomal gains and losses—had
447 marked upregulation of the ISR (Fig. 5F and fig. S11C). These results are consistent with models
448 in which cell cycle checkpoints are activated by the secondary consequences of aneuploidy (e.g.,
449 DNA damage or proteostatic stress) rather than changes in chromosome number *per se* (*45*, *46*).

450 Finally, we looked across all perturbations to systematically identify genetic drivers of
451 CIN. We assigned a score to each perturbation based on the average magnitude of induced
452 karyotypic abnormalities. Validating our approach, we found that CIN scores were strongly
453 correlated across K562 and RPE1 cell lines (r=0.69) and identified many known regulators of
454 chromosomal segregation, including components of the spindle assembly checkpoint, centromere,
455 and NDC80 complex (Fig. 5G). Remarkably, we uncovered CIN regulators with diverse cellular
456 roles, from cytoskeletal components to DNA repair machinery (Fig. 5H; table S4-S6). While many
457 of these genes have previously been associated with chromosomal instability through targeted
458 studies, the scale and single-cell resolution of Perturb-seq allowed us to identify numerous genetic
459 drivers of CIN in a single experiment. This analysis also shows the potential of single-cell CRISPR
460 screens to dissect phenotypes that were not predefined endpoints of the experiment.
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465 ***Discovery of stress-specific regulation of the mitochondrial genome***

466

467 Mitochondria arose from the engulfment and endosymbiotic evolution of an ancestral
468 alphaproteobacterium by the precursor to eukaryotic cells (*47*). While the vast majority (~99%) of
469 mitochondrially-localized proteins are encoded in the nuclear genome, mitochondria contain a
470 small (~16.6 kilobase) remnant of their ancestral genome encoding 2 rRNAs, 22 tRNAs, and 13
471 protein-coding genes in humans. An open question is how the nuclear and mitochondrial genomes
472 coordinate their expression to cope with mitochondrial stress (*48*). The scale of our experiment
473 provided a unique opportunity to investigate this question.

474 We began by comparing the nuclear transcriptional responses to CRISPRi-based depletion
475 of nuclear-encoded mitochondrial genes (i.e., mitochondrial perturbations). We found that
476 mitochondrial perturbations elicited relatively homogeneous nuclear transcriptional responses,
477 illustrated by well-correlated transcriptional phenotypes across mitochondrial perturbations (Fig.
478 6A and fig. S12A). While there was some variation in the magnitude of transcriptional responses
479 (e.g., proteostatic injury drove an especially strong ISR activation), nuclear transcriptional
480 responses generally failed to discriminate genetic perturbations by function. Although this result
481 was broadly consistent with recent literature that has highlighted the role of the ISR as response to
482 mitochondrial stress (*49–53*), the lack of functional specificity of the transcriptional response was
483 puzzling in light of: (i) the multifaceted roles of mitochondria in diverse processes such as
484 respiration, intermediary metabolism, iron-sulfur cluster biogenesis, and apoptosis and (ii) the
485 high-resolution separation of cytosolic perturbations by transcriptional response in our data
486 described above.

487 In contrast to the nuclear transcriptional response, we observed that the expression of
488 mitochondrially encoded genes was highly variable between different mitochondrial perturbations
489 (Fig. 6B; fig. S12B,C,D). When we clustered mitochondrial perturbations based solely on
490 expression levels of the 13 mitochondrially encoded genes, a remarkably intricate and coherent
491 pattern emerged: the clustering separated perturbations to Complex I, Complex IV, Complex III,
492 Complex V (ATP synthase), the mitochondrial large ribosomal subunit, the mitochondrial small
493 ribosomal subunit, chaperones/import machinery, and RNA processing factors (Fig. 6C; fig.
494 S12E). To quantitatively support this observation, we trained a random forest classifier to
495 distinguish cells with perturbations to different mitochondrial complexes and found that the
496 mitochondrial transcriptome was far more predictive than the nuclear transcriptome

497 (mitochondrial accuracy 0.64; nuclear accuracy: 0.25) (fig. S12F). We then visualized the gene
498 expression signatures of a subset of representative perturbations (Fig. 6D). The coregulation of
499 mitochondrial genes tended to reflect function, with the exception of the bicistronic mRNAs
500 *ND4L/ND4* and *ATP8/ATP6* (*54*). However, we did not identify a simple logic to explain the
501 connection between genetic perturbations to their observed transcriptional consequences. While
502 previous studies have described distinct regulation of the mitochondrial genome in response to
503 specific perturbations [notably, related to loss of complex III and complex IV assembly factors
504 (*55*, *56*)], our data generalize this phenomenon to a comprehensive set of stressors.

505 Next, we wanted to shed light on the mechanistic basis for this unappreciated complexity
506 of mitochondrial genome responses. Given its singular origin, the mitochondrial genome is
507 expressed by unique processes (Fig. 7A) (*57*). Mitochondrially encoded genes are transcribed as
508 part of three polycistronic transcripts punctuated by tRNAs. These transcripts are then processed
509 into rRNAs and mRNAs by tRNA excision, and individual mRNAs can be polyadenylated,
510 expressed, or degraded. This complex system limits the potential for distinct transcriptional control
511 but presents multiple opportunities for post-transcriptional regulation. To identify modes of
512 perturbation-elicited differential expression, we examined the distribution of Perturb-seq reads
513 along the mitochondrial genome (Fig. 7B). As our scRNA-seq used poly-A selection, most reads
514 aligned to the 3' ends of mRNAs. To validate the utility of this position-based analysis, we
515 confirmed that knockdown of known regulators of mitochondrial transcription (*TEFM*) and RNA
516 degradation (*PNPT1*) led to major shifts in the position of reads along the mitochondrial genome.
517 By contrast, many of the perturbation-specific responses discovered in the present study appeared
518 to cause shifts in the relative abundance of mRNAs rather than gross shifts in positional
519 alignments. To determine whether the observed mitochondrial genome responses reflected
520 regulation of the total level of mitochondrial mRNAs or specific regulation of mRNA
521 polyadenylation, we performed a bulk RNA-sequencing experiment with no poly-A selection. We
522 observed perturbation-specific changes in the level of total RNA similar to those measured by
523 scRNA-seq (cophenetic correlation r=0.79; Fig. 7C). Given the complexity of the observed
524 responses, we propose that there are likely to be multiple mechanisms that impact the levels of the
525 various mitochondrially encoded transcripts in response to different stressors.

526 Finally, we asked whether we could use the detailed clustering produced by the
527 mitochondrial genome to predict gene function. Knockdown of an unannotated gene, *TMEM242*,
528 produced a transcriptional signature resembling loss of ATP synthase in both K562 and RPE1 cells

529 (Fig. 7D; fig. S12G). Supporting this relationship, the top five co-essential genes with *TMEM242*
530 were components of ATP synthase in the Cancer Dependency Map. Using a Seahorse assay, we
531 further confirmed that basal respiration was decreased in *TMEM242* knockdown cells (Fig. 7E).
532 While this work was in progress, another group used a biochemical approach to show that
533 *TMEM242* physically interacts with ATP synthase subunits and regulates ATP synthase complex
534 assembly in cells (*58*). Together, these experiments discover a novel factor required for ATP
535 synthase activity and point to the precision of mitochondrial genome regulation.
536
537 **Discussion**
538
539 Single-cell CRISPR screens represent an emerging tool to generate rich genotype-
540 phenotype maps. However, to date, their use has been limited to the study of preselected genes
541 focused on discrete, predefined biological questions. Here, we perform genome-scale single-cell
542 CRISPR screens using Perturb-seq and demonstrate how these screens enable data-driven
543 dissection of a breadth of complex biological phenomena. Reflecting on this study, we highlight
544 key biological insights and derive principles to guide future discoveries from rich genotype-
545 phenotype maps.
546 A primary aim of large-scale functional screens is to organize genes into pathways or
547 complexes. To this end, we used Perturb-seq to perform high-resolution clustering of genetic
548 perturbations. From a single assay, we recapitulated thousands of known relationships while also
549 assigning new, experimentally validated roles to genes involved in ribosome biogenesis or
550 translation (*CCDC86*, *CINP*, *SPATA5L1*, *ZNF236*, *C1orf131*, *SPOUT1*, *TMA16*, *NOPCHAP1*,
551 *NEPRO*), transcription (*C7orf26*), and respiration (*TMEM242*). However, other large-scale
552 experimental techniques, such as protein-protein interaction mapping, genetic interaction
553 mapping, and co-essentiality analysis, similarly group genes or proteins by function. How then are
554 single-cell CRISPR screens distinct?
555 We argue that these screens are particularly powerful because of the intrinsic
556 interpretability of comprehensive genotype-phenotype maps, enabling in-depth dissection of the
557 functional consequences of genetic perturbations that impinge on many distinct aspects of cell
558 biology. Of particular note is the ability to use the information-rich readouts to study complex,
559 composite phenotypes, which are difficult to measure by other modalities. These composite
560 phenotypes can be created in a data-driven (e.g., deriving transcriptional programs) or hypothesis-

561 driven manner (e.g., measuring intron/exon ratios to study splicing), resulting in an enormous
562 breadth of measured phenotypes. In the case of scRNA-seq, we show that it measures not only
563 features such as differential gene expression and the activity of critical transcriptional programs,
564 but also RNA splicing and processing, expression of transposable elements, differentiation,
565 transcriptional heterogeneity, cell cycle progression, and chromosomal instability. Once a
566 phenotype is defined, the genotype-phenotype map can be used to explore its genetic
567 underpinnings, in a manner analogous to a forward genetic screen, as well as its relationship to
568 other cellular phenotypes.

569 An illustrative example of this process is our study of chromosomal instability. Based on
570 an initial observation of heterogeneous responses to specific perturbations, we suspected that some
571 cells carried genetically-induced chromosomal gains or losses. In a hypothesis-driven manner, we
572 then used our rich phenotypic data to discover a large collection of perturbations—which were
573 only loosely connected by clustering on average transcriptional phenotypes—that promote
574 chromosomal instability. Importantly, the single-cell nature of our Perturb-seq data also allowed
575 us to explore the relationship between karyotypic changes and other phenotypes, including cell
576 cycle progression and stress induction. While aneuploidy is an important hallmark of most cancers,
577 it has not been easy to study with traditional genetic screens as it requires both a single-cell and
578 multimodal readout. In future work, this platform could be used to investigate interactions between
579 genetic perturbations and specific karyotypes, karyotype-dependent stress responses, or the
580 temporal evolution of karyotypes (*59*).

581 Genetic perturbations can push cells into extreme states that are not observed in
582 unperturbed cells. Because composite phenotypes can be generated and explored without being
583 preregistered at the time of data collection, rich genotype-phenotype maps provide a powerful
584 resource for the discovery of new cellular behaviors. Using this ability, we discovered a remarkable
585 degree of stress-specific changes in the expression of mitochondrially encoded transcripts. It was
586 only possible to appreciate the functional specificity of this regulation by pairing a defined set of
587 mitochondrial perturbations with a high-dimensional readout. This discovery suggests a
588 framework to explain how cells cope with diverse insults to mitochondria: a general nuclear
589 response is layered over perturbation-specific changes in the expression level of mitochondrially
590 encoded genes (Fig. 7F). Building on this observation, we can ask new questions about the
591 mitochondrial stress response. The transcriptional changes we observed may reflect adaptive
592 responses or, alternatively, complex patterns of dysfunction owing to disruption of the intricate

593 system of mitochondrial gene expression. Understanding how and in what contexts this regulation
594 is adaptive may have important implications for diseases associated with mitochondrial stress. An
595 intriguing additional question is whether individual mitochondria are able to regulate their
596 expression autonomously. Combined with the nuanced responses observed here, this would
597 support and substantially extend the "co-location for redox regulation" (CoRR) hypothesis which
598 holds that the endosymbiotically derived mitochondrial genome has been retained through
599 evolution to enable localized regulation of mitochondrial gene expression (*60*).

600 A final theme emerging from our work is the flexibility of single-cell CRISPR screens
601 compared to other functional genomic approaches. Because these screens extract rich information
602 from each cell in a pooled format, they require only a fraction of the number of cells used by other
603 approaches and thus are well suited to the study of iPSC-derived cells and *in vivo* samples. As
604 technologies for single-cell, multimodal phenotyping advance, single-cell screens will continue to
605 become more powerful. At present, the major limitation of single-cell CRISPR screens is cost.
606 Careful experimental designs, such as multiplexed libraries or compressed sensing (*61*), together
607 with advances in single-cell phenotyping (*62*, *63*) and DNA sequencing promise to greatly increase
608 the scale of these experiments.

609 In sum, our study presents a blueprint for the construction and analysis of rich genotype-
610 phenotype maps to serve as a driving force for the systematic exploration of genetic and cellular
611 function.
612

613 **Materials and Methods**

614 A complete description of our Material and Methods is found in the Supplementary Material
615 online. This includes methods experimental methods related to Perturb-seq screens and
616 functional experiments, as well as computational methods detailing all data analysis.

617

618 **References and Notes**

619

620 1. J. G. Camp, R. Platt, B. Treutlein, Mapping human cell phenotypes to genotypes with single-
621 cell genomics. *Sci New York N Y*. 365, 1401–1405 (2019).

622 2. J. G. Doench, Am I ready for CRISPR? A user's guide to genetic screens. *Nat Rev Genet*. 19,
623 67–80 (2018).

624 3. N. J. Kramer, M. S. Haney, D. W. Morgens, A. Jovičić, J. Couthouis, A. Li, J. Ousey, R. Ma,
625 G. Bieri, C. K. Tsui, Y. Shi, N. T. Hertz, M. Tessier-Lavigne, J. K. Ichida, M. C. Bassik, A. D.
626 Gitler, CRISPR–Cas9 screens in human cells and primary neurons identify modifiers of
627 C9ORF72 dipeptide-repeat-protein toxicity. *Nat Genet*. 50, 603–612 (2018).

628 4. A. Tsherniak, F. Vazquez, P. G. Montgomery, B. A. Weir, G. Kryukov, G. S. Cowley, S. Gill,
629 W. F. Harrington, S. Pantel, J. M. Krill-Burger, R. M. Meyers, L. Ali, A. Goodale, Y. Lee, G.
630 Jiang, J. Hsiao, W. F. J. Gerath, S. Howell, E. Merkel, M. Ghandi, L. A. Garraway, D. E. Root,
631 T. R. Golub, J. S. Boehm, W. C. Hahn, Defining a Cancer Dependency Map. *Cell*. 170, 564-
632 576.e16 (2017).

633 5. T. Wang, K. Birsoy, N. W. Hughes, K. M. Krupczak, Y. Post, J. J. Wei, E. S. Lander, D. M.
634 Sabatini, Identification and characterization of essential genes in the human genome. *Science*.
635 350, 1096–1101 (2015).

636 6. E. Wang, H. Zhou, B. Nadorp, G. Cayanan, X. Chen, A. H. Yeaton, S. Nomikou, M. T.
637 Witkowski, S. Narang, A. Kloetgen, P. Thandapani, N. Ravn-Boess, A. Tsirigos, I. Aifantis,
638 Surface antigen-guided CRISPR screens identify regulators of myeloid leukemia differentiation.
639 *Cell Stem Cell*. 28, 718-731.e6 (2021).

640 7. T. Stuart, R. Satija, Integrative single-cell analysis. *Nat Rev Genet*. 20, 257–272 (2019).

641 8. L. Przybyla, L. A. Gilbert, A new era in functional genomics screens. *Nat Rev Genet*, 1–15
642 (2021).

643 9. B. Adamson, T. M. Norman, M. Jost, M. Y. Cho, J. K. Nuñez, Y. Chen, J. E. Villalta, L. A.
644 Gilbert, M. A. Horlbeck, M. Y. Hein, R. A. Pak, A. N. Gray, C. A. Gross, A. Dixit, O. Parnas, A.
645 Regev, J. S. Weissman, A Multiplexed Single-Cell CRISPR Screening Platform Enables
646 Systematic Dissection of the Unfolded Protein Response. *Cell*. 167, 1867-1882.e21 (2016).

647 10. A. Dixit, O. Parnas, B. Li, J. Chen, C. P. Fulco, L. Jerby-Arnon, N. D. Marjanovic, D.
648 Dionne, T. Burks, R. Raychowdhury, B. Adamson, T. M. Norman, E. S. Lander, J. S. Weissman,
649 N. Friedman, A. Regev, Perturb-Seq: Dissecting Molecular Circuits with Scalable Single-Cell
650 RNA Profiling of Pooled Genetic Screens. *Cell*. 167, 1853-1866.e17 (2016).

651 11. D. A. Jaitin, A. Weiner, I. Yofe, D. Lara-Astiaso, H. Keren-Shaul, E. David, T. M. Salame,
652 A. Tanay, A. van Oudenaarden, I. Amit, Dissecting Immune Circuits by Linking CRISPR-
653 Pooled Screens with Single-Cell RNA-Seq. *Cell*. 167, 1883-1896.e15 (2016).

654 12. P. Datlinger, A. F. Rendeiro, C. Schmidl, T. Krausgruber, P. Traxler, J. Klughammer, L. C.
655 Schuster, A. Kuchler, D. Alpar, C. Bock, Pooled CRISPR screening with single-cell
656 transcriptome readout. *Nat Methods*. 14, 297–301 (2017).

657 13. J. M. Replogle, T. M. Norman, A. Xu, J. A. Hussmann, J. Chen, J. Z. Cogan, E. J. Meer, J.
658 M. Terry, D. P. Riordan, N. Srinivas, I. T. Fiddes, J. G. Arthur, L. J. Alvarado, K. A. Pfeiffer, T.
659 S. Mikkelsen, J. S. Weissman, B. Adamson, Combinatorial single-cell CRISPR screens by direct
660 guide RNA capture and targeted sequencing. *Nat Biotechnol*. 38, 954–961 (2020).

661 14. A. J. Rubin, K. R. Parker, A. T. Satpathy, Y. Qi, B. Wu, A. J. Ong, M. R. Mumbach, A. L. Ji,
662 D. S. Kim, S. W. Cho, B. J. Zarnegar, W. J. Greenleaf, H. Y. Chang, P. A. Khavari, Coupled
663 Single-Cell CRISPR Screening and Epigenomic Profiling Reveals Causal Gene Regulatory
664 Networks. *Cell*. 176, 361-376.e17 (2019).

665 15. D. Feldman, A. Singh, J. L. Schmid-Burgk, R. J. Carlson, A. Mezger, A. J. Garrity, F. Zhang,
666 P. C. Blainey, Optical Pooled Screens in Human Cells. *Cell*. 179, 787-799.e17 (2019).

667 16. E. P. Mimitou, A. Cheng, A. Montalbano, S. Hao, M. Stoeckius, M. Legut, T. Roush, A.
668 Herrera, E. Papalexi, Z. Ouyang, R. Satija, N. E. Sanjana, S. B. Koralov, P. Smibert, Multiplexed
669 detection of proteins, transcriptomes, clonotypes and CRISPR perturbations in single cells. *Nat*
670 *Methods*. 16, 409–412 (2019).

671 17. C. J. Frangieh, J. C. Melms, P. I. Thakore, K. R. Geiger-Schuller, P. Ho, A. M. Luoma, B.
672 Cleary, L. Jerby-Arnon, S. Malu, M. S. Cuoco, M. Zhao, C. R. Ager, M. Rogava, L. Hovey, A.
673 Rotem, C. Bernatchez, K. W. Wucherpfennig, B. E. Johnson, O. Rozenblatt-Rosen, D.
674 Schadendorf, A. Regev, B. Izar, Multimodal pooled Perturb-CITE-seq screens in patient models
675 define mechanisms of cancer immune evasion. *Nat Genet*. 53, 332–341 (2021).

676 18. E. Papalexi, E. P. Mimitou, A. W. Butler, S. Foster, B. Bracken, W. M. Mauck, H.-H.
677 Wessels, Y. Hao, B. Z. Yeung, P. Smibert, R. Satija, Characterizing the molecular regulation of
678 inhibitory immune checkpoints with multimodal single-cell screens. *Nat Genet*. 53, 322–331
679 (2021).

680 19. A. Tanay, A. Regev, Scaling single-cell genomics from phenomenology to mechanism.
681 *Nature*. 541, 331–338 (2017).

682 20. T. M. Norman, M. A. Horlbeck, J. M. Replogle, A. Y. Ge, A. Xu, M. Jost, L. A. Gilbert, J. S.
683 Weissman, Exploring genetic interaction manifolds constructed from rich single-cell phenotypes.
684 *Science*. 365, 786–793 (2019).

685 21. A. H. Smits, F. Ziebell, G. Joberty, N. Zinn, W. F. Mueller, S. Clauder-Münster, D.
686 Eberhard, M. F. Savitski, P. Grandi, P. Jakob, A.-M. Michon, H. Sun, K. Tessmer, T.
687 Bürckstümmer, M. Bantscheff, L. M. Steinmetz, G. Drewes, W. Huber, Biological plasticity
688 rescues target activity in CRISPR knock outs. *Nat Methods*. 16, 1087–1093 (2019).

689 22. E. Haapaniemi, S. Botla, J. Persson, B. Schmierer, J. Taipale, CRISPR–Cas9 genome editing
690 induces a p53-mediated DNA damage response. *Nat Med*. 24, 927–930 (2018).

691 23. L. A. Gilbert, M. A. Horlbeck, B. Adamson, J. E. Villalta, Y. Chen, E. H. Whitehead, C.
692 Guimaraes, B. Panning, H. L. Ploegh, M. C. Bassik, L. S. Qi, M. Kampmann, J. S. Weissman,

693 Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. *Cell*. 159, 647–
694 661 (2014).

695 24. N. Alerasool, D. Segal, H. Lee, M. Taipale, An efficient KRAB domain for CRISPRi
696 applications in human cells. *Nat Methods*. 17, 1093–1096 (2020).

697 25. J. Rosenbluh, H. Xu, W. Harrington, S. Gill, X. Wang, F. Vazquez, D. E. Root, A. Tsherniak,
698 W. C. Hahn, Complementary information derived from CRISPR Cas9 mediated gene deletion
699 and suppression. *Nat Commun*. 8, 15403 (2017).

700 26. M. Giurgiu, J. Reinhard, B. Brauner, I. Dunger-Kaltenbach, G. Fobo, G. Frishman, C.
701 Montrone, A. Ruepp, CORUM: the comprehensive resource of mammalian protein complexes—
702 2019. *Nucleic Acids Res*. 47, D559–D563 (2019).

703 27. D. Szklarczyk, A. L. Gable, D. Lyon, A. Junge, S. Wyder, J. Huerta-Cepas, M. Simonovic,
704 N. T. Doncheva, J. H. Morris, P. Bork, L. J. Jensen, C. von Mering, STRING v11: protein–
705 protein association networks with increased coverage, supporting functional discovery in
706 genome-wide experimental datasets. *Nucleic Acids Res*. 47, D607–D613 (2019).

707 28. S. Singh, A. V. Broeck, L. Miller, M. Chaker-Margot, S. Klinge, Nucleolar maturation of the
708 human small subunit processome. *Science*. 373, eabj5338 (2021).

709 29. N. Kirstein, H. G. D. Santos, E. Blumenthal, R. Shiekhattar, The Integrator complex at the
710 crossroad of coding and noncoding RNA. *Curr Opin Cell Biol*. 70, 37–43 (2021).

711 30. I. Fianu, Y. Chen, C. Dienemann, O. Dybkov, A. Linden, H. Urlaub, P. Cramer, Structural
712 basis of Integrator-mediated transcription regulation. *Science*. 374, 883–887 (2021).

713 31. H. Zheng, Y. Qi, S. Hu, X. Cao, C. Xu, Z. Yin, X. Chen, Y. Li, W. Liu, J. Li, J. Wang, G.
714 Wei, K. Liang, F. X. Chen, Y. Xu, Identification of Integrator-PP2A complex (INTAC), an RNA
715 polymerase II phosphatase. *Sci New York N Y*. 370 (2020), doi:10.1126/science.abb5872.

716 32. M. M. Pfleiderer, W. P. Galej, Structure of the catalytic core of the Integrator complex. *Mol*
717 *Cell*. 81, 1246-1259.e8 (2021).

718 33. K. Sabath, M. L. Stäubli, S. Marti, A. Leitner, M. Moes, S. Jonas, INTS10–INTS13–INTS14
719 form a functional module of Integrator that binds nucleic acids and the cleavage module. *Nat*
720 *Commun*. 11, 3422 (2020).

721 34. S. K. Radhakrishnan, C. S. Lee, P. Young, A. Beskow, J. Y. Chan, R. J. Deshaies,
722 Transcription Factor Nrf1 Mediates the Proteasome Recovery Pathway after Proteasome
723 Inhibition in Mammalian Cells. *Mol Cell*. 38, 17–28 (2010).

724 35. A. Mamińska, A. Bartosik, M. Banach-Orłowska, I. Pilecka, K. Jastrzębski, D. Zdżalik-
725 Bielecka, I. Castanon, M. Poulain, C. Neyen, L. Wolińska-Nizioł, A. Toruń, E. Szymańska, A.
726 Kowalczyk, K. Piwocka, A. Simonsen, H. Stenmark, M. Fürthauer, M. González-Gaitán, M.
727 Miaczynska, ESCRT proteins restrict constitutive NF-κB signaling by trafficking cytokine
728 receptors. *Sci Signal*. 9, ra8–ra8 (2016).

729 36. J. Luo, H. Yang, B.-L. Song, Mechanisms and regulation of cholesterol homeostasis. *Nat Rev*
730 *Mol Cell Bio*. 21, 225–245 (2020).

731 37. J. F. Leary, B. M. Ohlsson-Wilhelm, R. Giuliano, S. Labella, B. Farley, P. T. Rowley,
732 Multipotent human hematopoietic cell line K562: Lineage-specific constitutive and inducible
733 antigens. *Leukemia Res*. 11, 807–815 (1987).

734 38. S. H. Orkin, L. I. Zon, Hematopoiesis: An Evolving Paradigm for Stem Cell Biology. *Cell*.
735 132, 631–644 (2008).

736 39. L. Yu, G. Myers, C.-J. Ku, E. Schneider, Y. Wang, S. A. Singh, N. Jearawiriyapaisarn, A.
737 White, T. Moriguchi, R. Khoriaty, M. Yamamoto, M. G. Rosenfeld, J. Pedron, J. H. Bushweller,
738 K.-C. Lim, J. D. Engel, An erythroid-to-myeloid cell fate conversion is elicited by LSD1
739 inactivation. *Blood*. 138, 1691–1704 (2021).

740 40. T. Maes, C. Mascaró, I. Tirapu, A. Estiarte, F. Ciceri, S. Lunardi, N. Guibourt, A. Perdones,
741 M. M. P. Lufino, T. C. P. Somervaille, D. H. Wiseman, C. Duy, A. Melnick, C. Willekens, A.
742 Ortega, M. Martinell, N. Valls, G. Kurz, M. Fyfe, J. C. Castro-Palomino, C. Buesa, ORY-1001, a
743 Potent and Selective Covalent KDM1A Inhibitor, for the Treatment of Acute Leukemia. *Cancer*
744 *Cell*. 33, 495-511.e12 (2018).

745 41. B. Sharma, L. Xie, F. Yang, W. Wang, Q. Zhou, M. Xiang, S. Zhou, W. Lv, Y. Jia, L.
746 Pokhrel, J. Shen, Q. Xiao, L. Gao, W. Deng, Recent advance on PTP1B inhibitors and their
747 biomedical applications. *Eur J Med Chem*. 199, 112376 (2020).

748 42. A. Musacchio, E. D. Salmon, The spindle-assembly checkpoint in space and time. *Nat Rev*
749 *Mol Cell Bio*. 8, 379–393 (2007).

750 43. A. P. Patel, I. Tirosh, J. J. Trombetta, A. K. Shalek, S. M. Gillespie, H. Wakimoto, D. P.
751 Cahill, B. V. Nahed, W. T. Curry, R. L. Martuza, D. N. Louis, O. Rozenblatt-Rosen, M. L. Suvà,
752 A. Regev, B. E. Bernstein, Single-cell RNA-seq highlights intratumoral heterogeneity in primary
753 glioblastoma. *Science*. 344, 1396–1401 (2014).

754 44. N. Jelluma, A. B. Brenkman, N. J. F. van den Broek, C. W. A. Cruijsen, M. H. J. van Osch,
755 S. M. A. Lens, R. H. Medema, G. J. P. L. Kops, Mps1 Phosphorylates Borealin to Control
756 Aurora B Activity and Chromosome Alignment. *Cell*. 132, 233–246 (2008).

757 45. S. Santaguida, A. Richardson, D. R. Iyer, O. M'Saad, L. Zasadil, K. A. Knouse, Y. L. Wong,
758 N. Rhind, A. Desai, A. Amon, Chromosome Mis-segregation Generates Cell-Cycle-Arrested
759 Cells with Complex Karyotypes that Are Eliminated by the Immune System. *Dev Cell*. 41, 638-
760 651.e5 (2017).

761 46. S. Santaguida, A. Amon, Short- and long-term effects of chromosome mis-segregation and
762 aneuploidy. *Nat Rev Mol Cell Bio*. 16, 473–485 (2015).

763 47. J. R. Friedman, J. Nunnari, Mitochondrial form and function. *Nature*. 505, 335–343 (2014).

764 48. P. M. Quirós, A. Mottis, J. Auwerx, Mitonuclear communication in homeostasis and stress.
765 *Nat Rev Mol Cell Biology*. 17, 213–26 (2016).

766 49. E. Mick, D. V. Titov, O. S. Skinner, R. Sharma, A. A. Jourdain, V. K. Mootha, Distinct
767 mitochondrial defects trigger the integrated stress response depending on the metabolic state of
768 the cell. *Elife*. 9, e49178 (2020).

769 50. P. M. Quirós, M. A. Prado, N. Zamboni, D. D'Amico, R. W. Williams, D. Finley, S. P. Gygi,
770 J. Auwerx, Multi-omics analysis identifies ATF4 as a key regulator of the mitochondrial stress
771 response in mammals. *J Cell Biol*. 216, 2027–2045 (2017).

772 51. C. Münch, J. W. Harper, Mitochondrial unfolded protein response controls matrix pre-RNA
773 processing and translation. *Nature*. 534, 710–713 (2016).

774 52. E. Fessler, E.-M. Eckl, S. Schmitt, I. A. Mancilla, M. F. Meyer-Bender, M. Hanf, J.
775 Philippou-Massier, S. Krebs, H. Zischka, L. T. Jae, A pathway coordinated by DELE1 relays
776 mitochondrial stress to the cytosol. *Nature*. 579, 433–437 (2020).

777 53. X. Guo, G. Aviles, Y. Liu, R. Tian, B. A. Unger, Y.-H. T. Lin, A. P. Wiita, K. Xu, M. A.
778 Correia, M. Kampmann, Mitochondrial stress is relayed to the cytosol by an OMA1-DELE1-HRI
779 pathway. *Nature*. 579, 427–432 (2020).

780 54. T. R. Mercer, S. Neph, M. E. Dinger, J. Crawford, M. A. Smith, A.-M. J. Shearwood, E.
781 Haugen, C. P. Bracken, O. Rackham, J. A. Stamatoyannopoulos, A. Filipovska, J. S. Mattick,
782 The Human Mitochondrial Transcriptome. *Cell*. 146, 645–658 (2011).

783 55. R. Richter-Dennerlein, S. Oeljeklaus, I. Lorenzi, C. Ronsör, B. Bareth, A. B. Schendzielorz,
784 C. Wang, B. Warscheid, P. Rehling, S. Dennerlein, Mitochondrial Protein Synthesis Adapts to
785 Influx of Nuclear-Encoded Protein. *Cell*. 167, 471-483.e10 (2016).

786 56. R. Salvatori, K. Kehrein, A. P. Singh, W. Aftab, B. V. Möller-Hergt, I. Forne, A. Imhof, M.
787 Ott, Molecular Wiring of a Mitochondrial Translational Feedback Loop. *Mol Cell*. 77, 887-
788 900.e5 (2020).

789 57. E. Kummer, N. Ban, Mechanisms and regulation of protein synthesis in mitochondria. *Nat*
790 *Rev Mol Cell Bio*. 22, 307–325 (2021).

791 58. J. Carroll, J. He, S. Ding, I. M. Fearnley, J. E. Walker, TMEM70 and TMEM242 help to
792 assemble the rotor ring of human ATP synthase and interact with assembly factors for complex I.
793 *P Natl Acad Sci Usa*. 118, e2100558118 (2021).

794 59. U. Ben-David, A. Amon, Context is everything: aneuploidy in cancer. *Nat Rev Genet*. 21,
795 44–62 (2020).

796 60. J. F. Allen, The CoRR hypothesis for genes in organelles. *J Theor Biol*. 434, 50–57 (2017).

797 61. B. Cleary, L. Cong, A. Cheung, E. S. Lander, A. Regev, Efficient Generation of
798 Transcriptomic Profiles by Random Composite Measurements. *Cell*. 171, 1424-1436.e18 (2017).

799 62. P. Datlinger, A. F. Rendeiro, T. Boenke, M. Senekowitsch, T. Krausgruber, D. Barreca, C.
800 Bock, Ultra-high-throughput single-cell RNA sequencing and perturbation screening with
801 combinatorial fluidic indexing. *Nat Methods*. 18, 635–642 (2021).

802 63. B. K. Martin, C. Qiu, E. Nichols, M. Phung, R. Green-Gladden, S. Srivatsan, R. Blecher-
803 Gonen, B. J. Beliveau, C. Trapnell, J. Cao, J. Shendure, An optimized protocol for single cell
804 transcriptional profiling by combinatorial indexing. *Arxiv* (2021).

805 64. M. Jost, Y. Chen, L. A. Gilbert, M. A. Horlbeck, L. Krenning, G. Menchon, A. Rai, M. Y.
806 Cho, J. J. Stern, A. E. Prota, M. Kampmann, A. Akhmanova, M. O. Steinmetz, M. E.
807 Tanenbaum, J. S. Weissman, Combined CRISPRi/a-Based Chemical Genetic Screens Reveal
808 that Rigosertib Is a Microtubule-Destabilizing Agent. *Mol Cell*. 68, 210-223.e6 (2017).

809 65. S. A. Lambert, A. Jolma, L. F. Campitelli, P. K. Das, Y. Yin, M. Albu, X. Chen, J. Taipale,
810 T. R. Hughes, M. T. Weirauch, The Human Transcription Factors. *Cell*. 172, 650–665 (2018).

811 66. M. A. Horlbeck, L. A. Gilbert, J. E. Villalta, B. Adamson, R. A. Pak, Y. Chen, A. P. Fields,
812 C. Y. Park, J. E. Corn, M. Kampmann, J. S. Weissman, Compact and highly active next-
813 generation libraries for CRISPR-mediated gene repression and activation. *Elife*. 5, e19760
814 (2016).

815 67. N. D. Elrod, T. Henriques, K.-L. Huang, D. C. Tatomer, J. E. Wilusz, E. J. Wagner, K.
816 Adelman, The Integrator Complex Attenuates Promoter-Proximal Transcription at Protein-
817 Coding Genes. *Mol Cell*. 76, 738-752.e7 (2019).

818 68. K.-L. Huang, D. Jee, C. B. Stein, N. D. Elrod, T. Henriques, L. G. Mascibroda, D. Baillat, W.
819 K. Russell, K. Adelman, E. J. Wagner, Integrator Recruits Protein Phosphatase 2A to Prevent
820 Pause Release and Facilitate Transcription Termination. *Mol Cell*. 80, 345-358.e9 (2020).

821 69. A. P. Anderson, X. Luo, W. Russell, Y. W. Yin, Oxidative damage diminishes mitochondrial
822 DNA polymerase replication fidelity. *Nucleic Acids Res*. 48, 817–829 (2020).

823 70. K. A. Reimer, C. A. Mimoso, K. Adelman, K. M. Neugebauer, Co-transcriptional splicing
824 regulates 3′ end cleavage during mammalian erythropoiesis. *Mol Cell*. 81, 998-1012.e7 (2021).

825 71. Z. Xie, A. Bailey, M. V. Kuleshov, D. J. B. Clarke, J. E. Evangelista, S. L. Jenkins, A.
826 Lachmann, M. L. Wojciechowicz, E. Kropiwnicki, K. M. Jagodnik, M. Jeon, A. Ma'ayan, Gene
827 Set Knowledge Discovery with Enrichr. *Curr Protoc*. 1, e90 (2021).

828 72. J. He, I. A. Babarinde, L. Sun, S. Xu, R. Chen, J. Shi, Y. Wei, Y. Li, G. Ma, Q. Zhuang, A.
829 P. Hutchins, J. Chen, Identifying transposable element expression dynamics and heterogeneity
830 during development at the single-cell level with a processing pipeline scTE. *Nat Commun*. 12,
831 1456 (2021).

832 73. P. Ma, M. W. Mahoney, B. Yu, A Statistical Perspective on Algorithmic Leveraging. *Arxiv*
833 (2013).

834 74. R. Satija, J. A. Farrell, D. Gennert, A. F. Schier, A. Regev, Spatial reconstruction of single-
835 cell gene expression data. *Nat Biotechnol*. 33, 495–502 (2015).

836

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872 Novartis not related to this work. MJ consults for Maze Therapeutics and Gate Bioscience. TMN
873 consults for Maze Therapeutics. JSW declares outside interest in 5 AM Venture, Amgen,
874 Chroma Medicine, KSQ Therapeutics, Maze Therapeutics, Tenaya Therapeutics, Tessera
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876 TMN, MJ, and JSW as inventors have filed patent applications related to CRISPRi/a screening
877 and Perturb-seq.
878
879 **Data and materials availability:** Raw sequencing data will be deposited into SRA. An interactive
880 data browser including processed, downloadable single-cell and pseudobulk populations will be
881 made available upon publication. Our previously published analytic framework for Perturb-seq
882 analysis is available at https://github.com/thomasmaxwellnorman/Perturbseq_GI. Scripts for
883 guide assignment are available at https://github.com/josephreplogle/guide_calling. Additional
884 code related to specific analyses will be made available on github upon publication.
885
886 **Supplementary Materials:**
887 Materials and Methods
888 Figs. S1 to S12
889 Tables S1 to S9

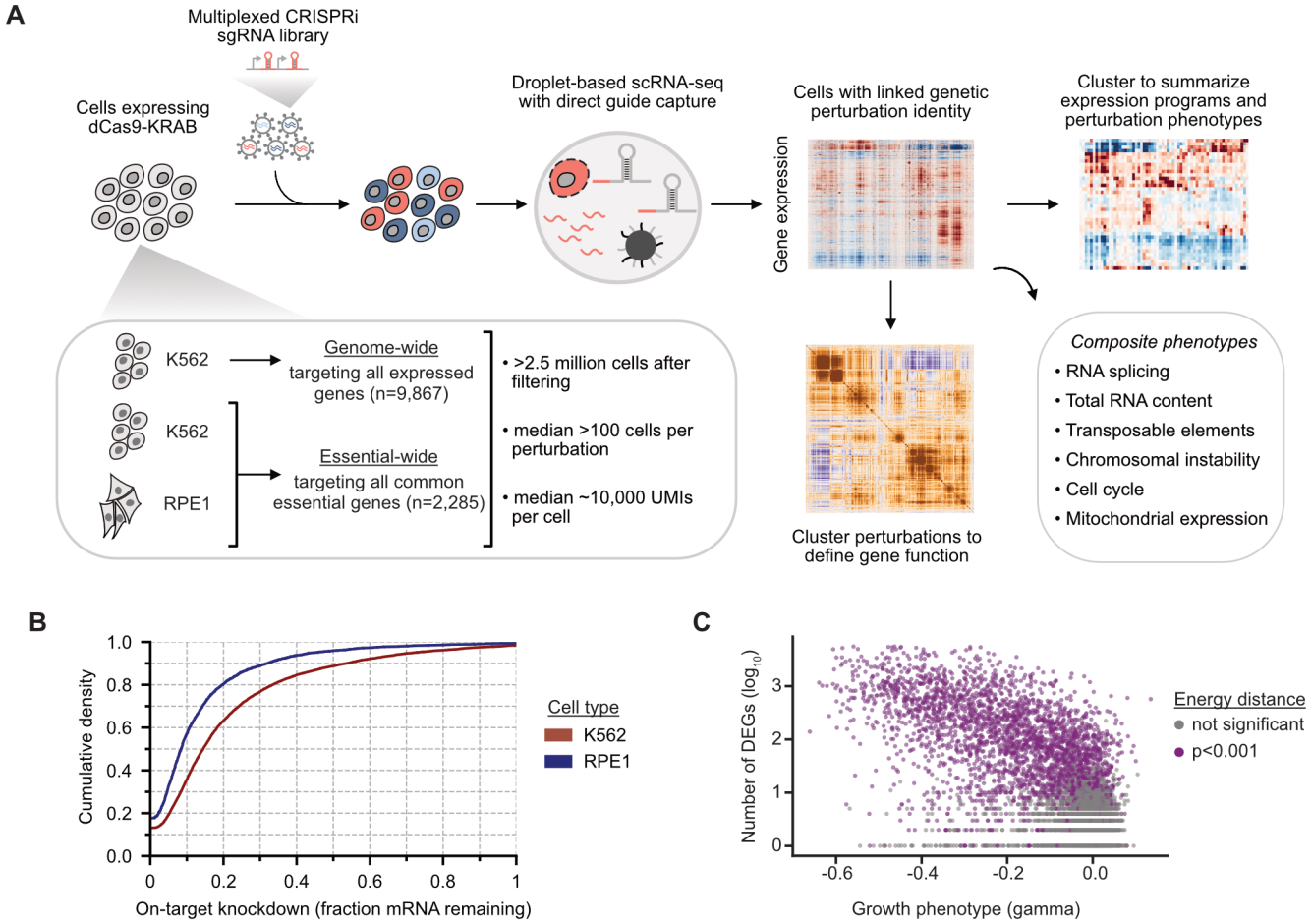

Figure 1: Genome-scale Perturb-seq via multiplexed CRISPRi. (A) Schematic experimental strategy. A multiplexed CRISPRi sgRNA library was used to knock down all expressed genes (in K562 cells) or all common essential genes (in RPE1 and K562 cells). Cells were transcriptionally profiled using droplet-based single-cell RNA-sequencing, with genetic perturbations assigned to cells by direct capture and sequencing of sgRNAs. **(B)** On-target knockdown statistics. Cumulative density plot of on-target knockdown, for n=9,464 target genes in K562 cells (red) and n=2,333 target genes in RPE1 cells (blue). **(C)** Comparing growth phenotype versus the number of differentially expressed genes (DEGs) for each multiplexed guide pair in K562 cells. Growth phenotypes are reported as the $\log_2$ guide enrichment per cell doubling (gamma). DEGs were determined using a two-sample Anderson-Darling test compared against non-targeting guides, and a pseudocount of 1 was added to the number of DEGs before $\log_{10}$ transformation. Dots are colored by Energy distance as either permutation significant (purple) or not significant (grey). The growth phenotype and number of DEGs are anticorrelated (Spearman's rho=-0.51).

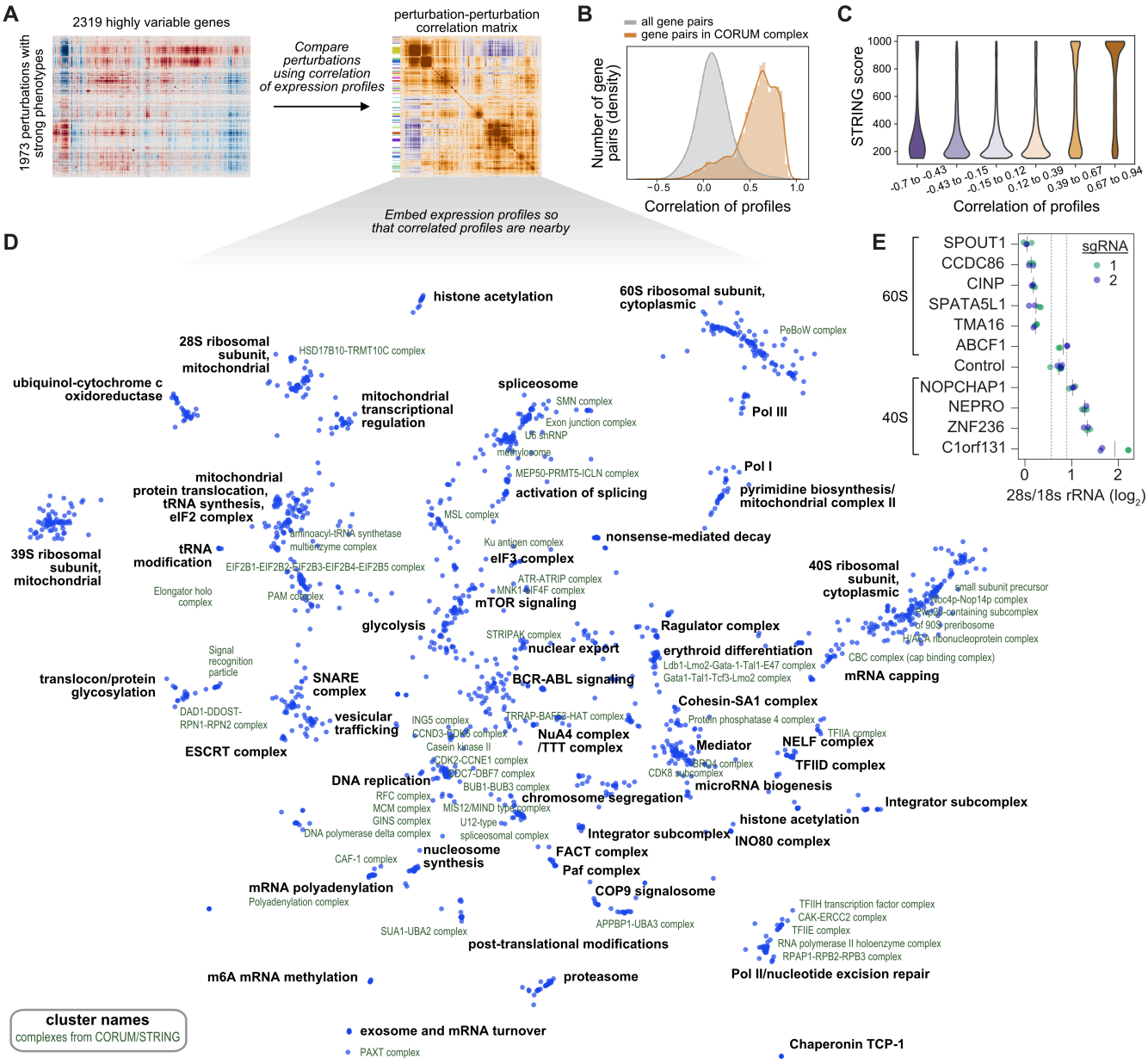

Figure 2: Data-driven inference of gene function from transcriptional phenotypes. (A) Schematic of analysis. To examine the ability of transcriptional phenotypes to assign gene function, we analyzed 1973 genetic perturbations that elicited strong responses. Perturbations were compared and clustered using the correlation of gene expression across 2319 highly variable genes. **(B)** Expression profile correlations among genes in curated complexes. 327 protein complexes from the CORUM3.0 database have at least two thirds of complex subunits within the dataset. Plot compares the distribution of pairwise expression profile correlations among genes in complexes vs. all possible gene-gene pairs. **(C)** Comparing expression profile correlations to predicted protein-protein interactions from STRING. 243,558 gene-gene relationships within the dataset are scored within STRING. The relationships were sorted into 6 equally spaced bins based on expression profile correlation. Plot shows kernel density estimates of STRING scores within each bin. **(D)** Minimum distortion embedding of dataset. Each dot represents a genetic perturbation, arranged so that perturbations with correlated expression profiles are nearby in the two dimensional embedding. Manual annotations (black labels) of

cluster function are placed near the median location of genes within the cluster. CORUM complexes or STRING clusters (green labels) are annotated when involved genes are nearby within the embedding. **(E)** Quantification of 28s to 18s rRNA ratio. Poorly characterized genes with Perturb-seq predicted roles in ribosome biogenesis were targeted by CRISPRi. The 28s/18s rRNA ratio was measured by Bioanalyzer electrophoresis in biological duplicate with two distinct sgRNAs per gene (green and blue; solid grey lines represent mean). Dotted grey lines represent two standard deviations above and below the mean of non-targeting controls.

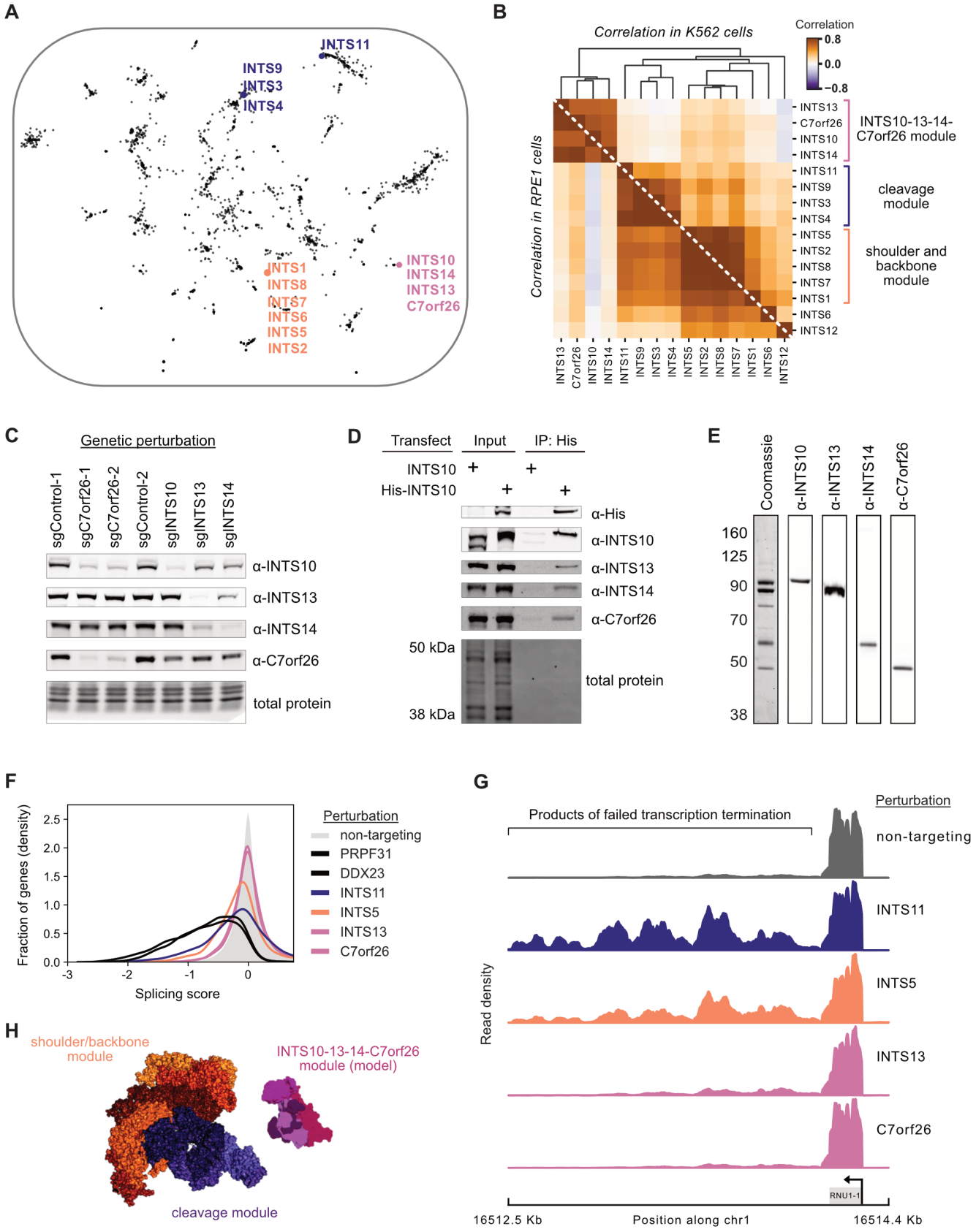

Figure 3. (legend on next page)

Figure 3: Perturb-seq discovers a novel gene member and functional submodules of the Integrator complex. (A) Location of known Integrator complex members in the minimum distortion embedding. **(B)** Relationship between Integrator complex members and *C7orf26* in K562 cells (top) and RPE1 cells (bottom). The heatmap displays the Pearson correlation between pseudobulk *z*-normalized gene expression profiles of Integrator complex members. Genetic perturbations are ordered by average linkage hierarchical clustering based on correlation in K562 cells. Functional modules suggested by the clustering are highlighted. **(C)** Co-depletion of Integrator complex members. Individual Integrator complex members were depleted in CRISPRi K562 cells. Lysates were then probed for other module members by western blot. **(D)** Co-immunoprecipitation of endogenous C7orf26 with His-INTS10. HEK293T were transfected with His-INTS10 or INTS10. Cell lysates were affinity purified and select Integrator proteins were probed by western blot. **(E)** Purification of a INTS10-INTS13-INTS14-C7orf26 complex. His-INTS10, INTS13, INTS14, and C7orf26 were overexpressed in Expi293 cells, affinity purified, and separated via SEC. The INTS10-INTS13-INTS14-C7orf26 proteins co-fractionated as visualized by Western blotting. **(F)** Effects of Integrator modules on splicing from Perturb-seq data. Histogram (kernel density estimate) compares gene-level splicing scores. Splicing scores represent the change in the $\log_2$ ratio of total to unspliced reads for each gene relative to non-targeting control guides. Representative genetic perturbations from Integrator modules as well as the spliceosome are shown colored by module. **(G)** Density of PRO-seq reads at the snRNA *RNU1-1* locus mapping actively engaged RNA polymerase II. For each perturbation, densities are shown relative to the maximum read count in the locus. **(H)** Structure of the Integrator complex colored by functional modules revealed by Perturb-seq. The endonuclease (blue) and shoulder/backbone (orange) modules were obtained from the cryo-EM structure (*31*). The model of the newly discovered 10-13-14-C7orf26 module was built by docking the crystal structure of INTS13-INTS14 (*33*) with an AlphaFold multimeric model of INTS10 and C7orf26.

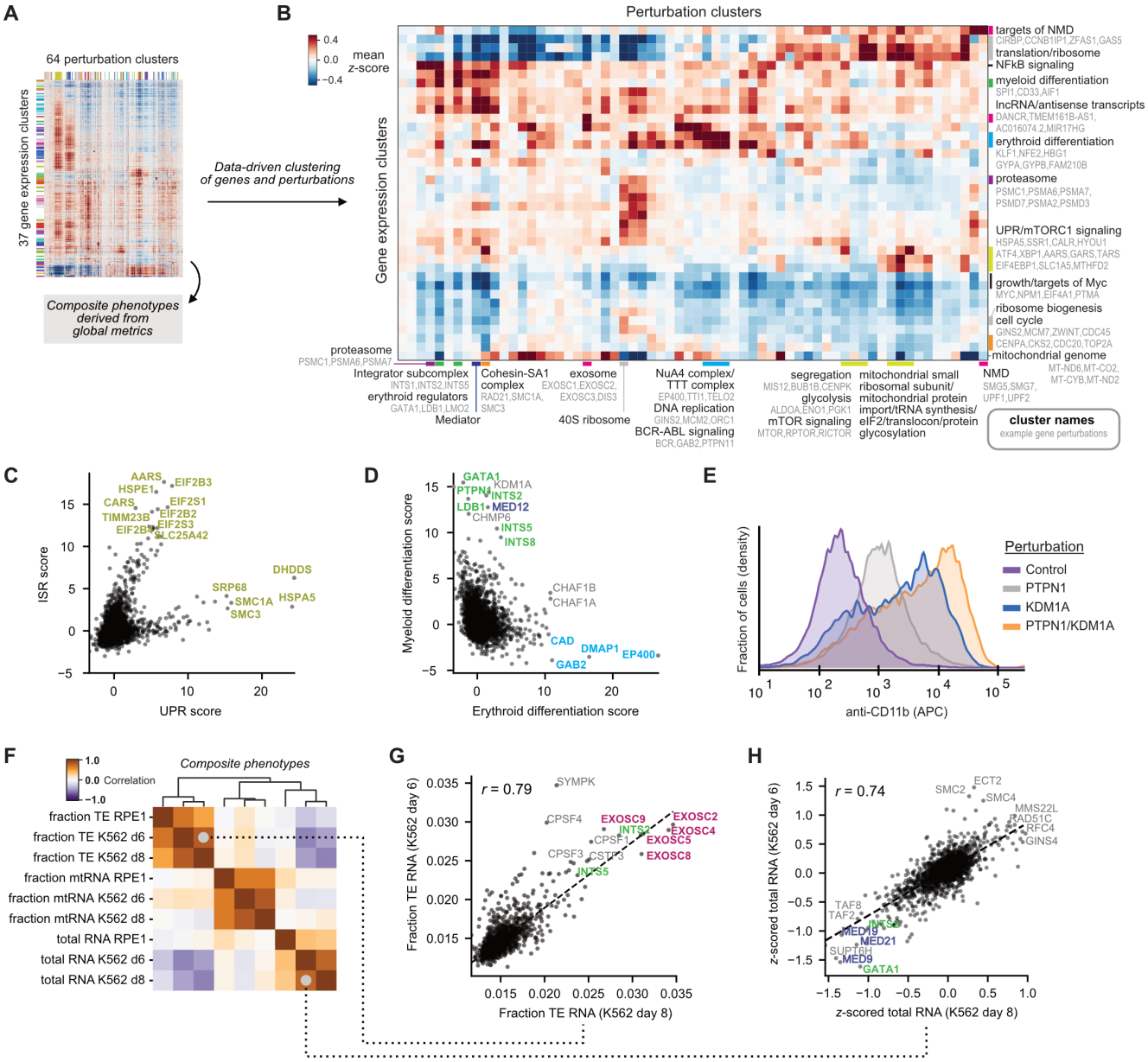

Figure 4: Summarizing genotype-phenotype relationships with Perturb-seq. (A) Schematic of analysis. To produce a high-level summary of genotype-phenotype relationships in K562 cells, 1973 genetic perturbations that elicited strong responses and 2319 highly variable genes were clustered using HDBSCAN after nonlinear embedding. Alternatively, composite phenotypes were derived from global metrics in a hypothesis-driven manner. **(B)** Heatmap of the high-level genotype-phenotype map. The heatmap represents the mean z-scored expression for gene expression and perturbation clusters. For a subset of clusters, clustered are labelled with manual annotations (black labels) of cluster function along with example genes within the cluster (light gray labels). **(C)** Comparison of ISR and UPR scores for genetic perturbations. Scores were recovered from unbiased clustering of genes by genetic dependency, and manually annotated. **(D)** Comparison of erythroid and myeloid differentiation scores for genetic perturbations. Scores were recovered from unbiased clustering of genes by genetic dependency, and manually annotated. Genetic perturbations are colored to reflect cluster identity. **(E)** Expression of CD11b/ITGAM in

K562 cells upon knockdown of *PTPN1* or *KDM1A*. CD11b was labelled by cell surface staining with anti-CD11b antibody and measured by flow cytometry. **(F)** Correlation of composite phenotypes across time points and cell types. Composite phenotypes were defined in a hypothesis-driven manner. Fraction TE (repetitive and transposable element) represents the number of non-intronic reads mapped to TEs over total, averaged over all cells bearing each perturbation (both collapsed on UMIs). Fraction mtRNA represents the mean number of reads mapped to mitochondrial genome protein-coding genes over total. Total RNA represents the mean total RNA content (number of UMIs). **(G)** Comparison of TE expression across time points. The mean fraction TE reads per perturbation is highly correlated across time points in K562 cells (r=0.79). Genetic perturbations are colored to reflect cluster identity. **(H)** Comparison of total RNA content across time points. Total RNA content per perturbation is highly correlated across time points in K562 cells (r=0.74). Genetic perturbations are colored to reflect cluster identity.

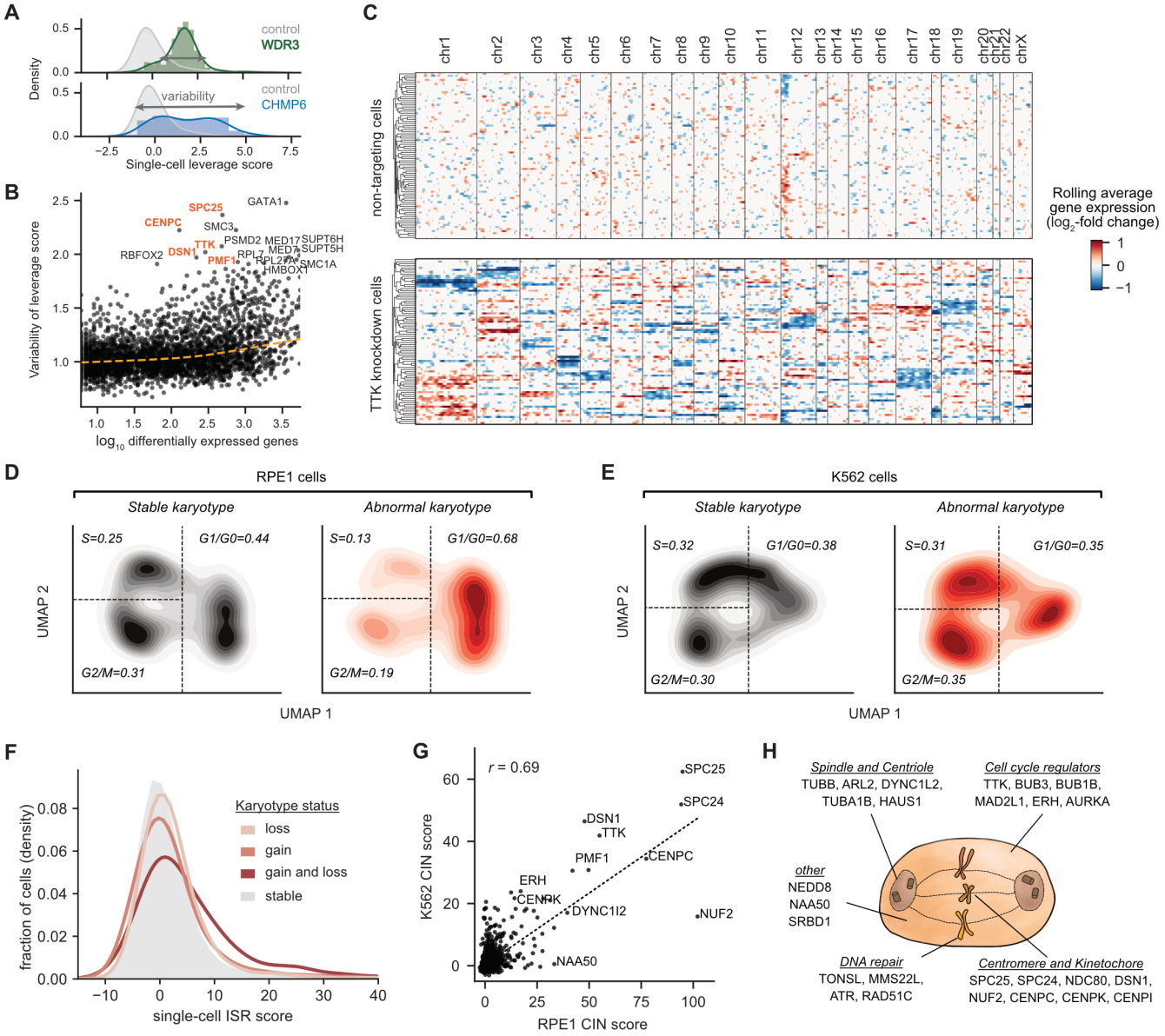

Figure 5: Exploring acute consequences and genetic drivers of aneuploidy in single-cells. (A) Schematic of heterogeneity statistic. Single-cell leverage scores quantify how outlying each cell is relative to non-targeting control cells by PCA. For each perturbation, heterogeneity of single-cell phenotypes is quantified as the standard deviation of leverage scores. **(B)** Identifying heterogeneous perturbations. Known regulators of chromosome segregation were among the perturbations with the highest single-cell heterogeneity (high variability of leverage scores), especially compared to their number of differentially expressed genes (based on Anderson-Darling test). **(C)** Heatmap of chromosomal copy number inference from Perturb-seq data. For all genes (expressed >0.05 UMI per cell), the log-fold change in expression is calculated with respect to the average of non-targeting control cells, and genes are ordered along the genome. A weighted moving average of 100 genes is used infer copy number changes (columns) in single-cells (rows) with noise and median filtering. 80 *TTK* knockdown RPE1 cells and 80 randomly sampled non-targeting control RPE1 cells are shown. Cells are ordered by average linkage hierarchical clustering based on correlation of chromosomal copy number profiles. **(D, E)** Comparison of cell

cycle occupancy upon acute karyotypic changes. Abnormal karyotypic cells were defined as having ≥1 chromosome with evidence of changes in chromosomal copy number for >80% of the chromosomal length. For single-cells, cell-cycle positioning was inferred by UMAP dimension reduction on differential expression profiles of 199 selected cell-cycle regulated genes. Cell cycle occupancy is shown as a 2D kernel density estimate of a random subset of 1000 cells per karyotypic status. Approximate gates between cell cycle phases (G1 or G0; S; G2 or M) are shown as dotted lines, and the fraction of cells in each cell cycle phase are indicated. **(F)** Effect of chromosomal instability (CIN) on activation of the Integrated Stress Response (ISR). Histogram (kernel density estimate) compares the ISR score versus CIN status in RPE1 cells. CIN status is defined as evidence of gain or loss of chromosomal copy number for >80% of the chromosomal length, with 240,768 stable cells, 5,522 cells bearing chromosomal loss, 1987 cells bearing chromosomal gain, and 904 cells bearing gain and loss of chromosomes. ISR score is defined as the sum of z-normalized expression of ISR marker genes where increased values indicate stronger ISR activation. **(G)** Comparison of the effect of genetic perturbations on the CIN score across cell types. For each genetic perturbation, the CIN score is calculated as the mean single-cell sum of squared CIN values, *z*-normalized relative to non-targeting control perturbations. The CIN score is correlated across cell types (r=0.69). **(H)** Schematic of a subset of genetic perturbations that drive CIN. CIN drivers play diverse roles in mitosis, cell cycle regulation, and DNA repair.

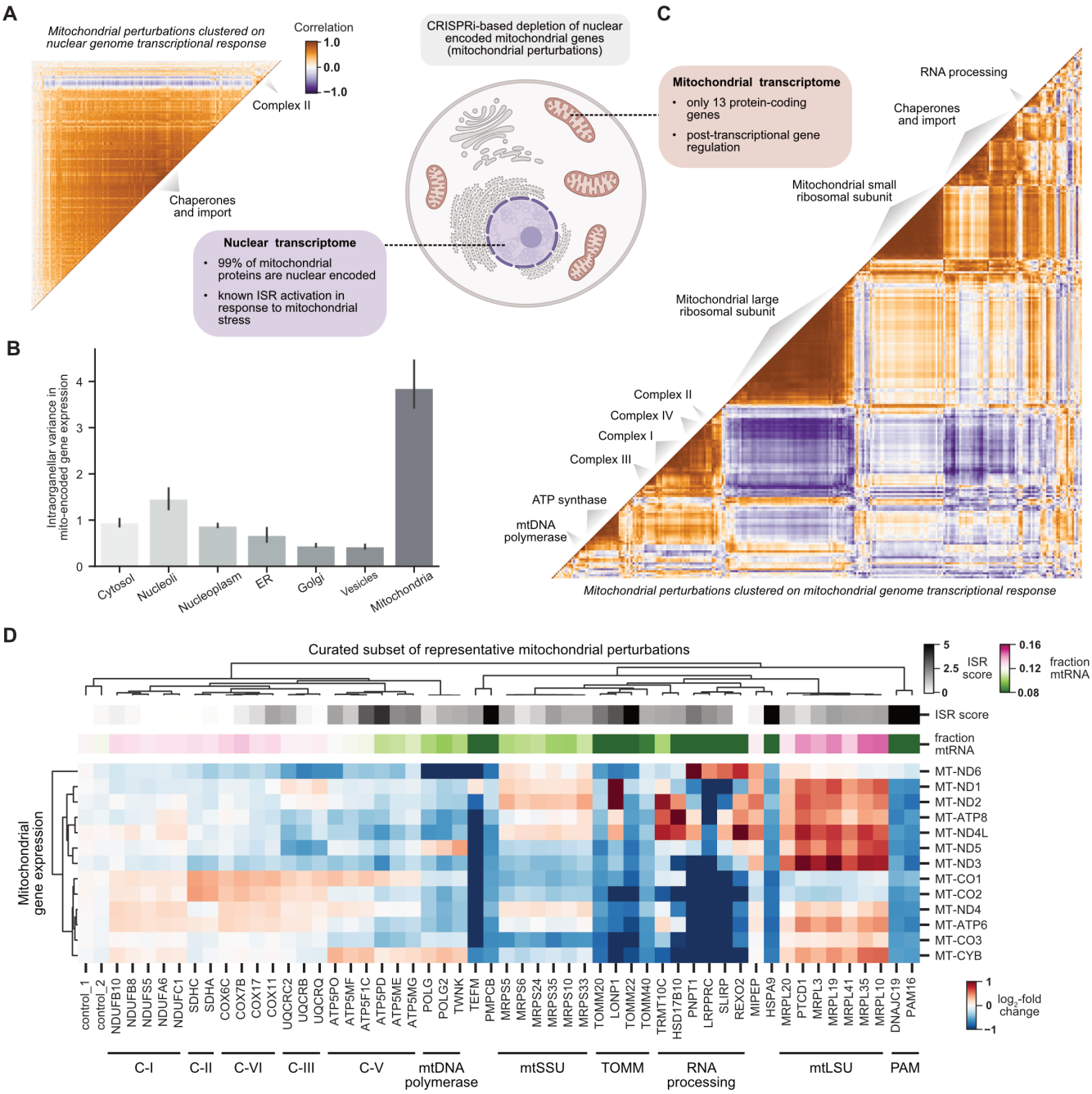

Figure 6: Global organization of the transcriptional response to mitochondrial stress. (A) Clustering mitochondrial perturbations by nuclear transcriptional response. CRISPRi enables knockdown of nuclear-encoded genes whose protein products are targeted to mitochondria (mitochondrial perturbations). Mitochondrial perturbations were annotated by MitoCarta3.0 and subset to those with a strong transcriptional phenotype (n=268 mitochondrial perturbations). Gene expression profiles were restricted to nuclear encoded genes which includes 99% of mitochondrial proteins. The heatmap displays the Pearson correlation between pseudobulk *z*-normalized gene expression profiles of mitochondrial perturbations in K562 cells. Genetic perturbations were clustered by HDBSCAN with a correlation metric. **(B)** Comparing variability in the mitochondrial transcriptome by perturbation localization. The mitochondrial genome encodes 13 protein-coding

genes. Genetic perturbations were grouped based on localization of their protein products as determined by the Human Protein Atlas. For each of these 13 mitochondrially encoded genes, the variance in pseudobulk z-normalized expression profiles was calculated between all perturbations with the same localization. Barplots represent the average across genes with 95% confidence interval obtained by bootstrapping. **(C)** Clustering mitochondrial perturbations by mitochondrial transcriptional response. Mitochondrial perturbations were annotated by MitoCarta3.0 and subset to those with a strong transcriptional phenotype as above (n=268 mitochondrial perturbations). Gene expression profiles were restricted to the 13 mitochondrial-encoded genes. The heatmap displays the Pearson correlation between pseudobulk *z*-normalized gene expression profiles of mitochondrial perturbations in K562 cells. Genetic perturbations are clustered by HDBSCAN with a correlation metric. Clusters were manually annotated. **(D)** Heatmap visualizing the mitochondrial genome transcriptional response to diverse mitochondrial stressors. The expression ($\log_2$ fold-change relative to non-targeting controls) of the 13 mitochondrially encoded genes is shown for a subset of perturbations representative of different mitochondrial complexes or function. Neither the ISR score nor mean fraction of mitochondrial RNA (mtRNA) would allow for high-resolution clustering by function as provided by the mitochondrial genome response. Genetic perturbations and genes are ordered by average linkage hierarchical clustering with a correlation metric.

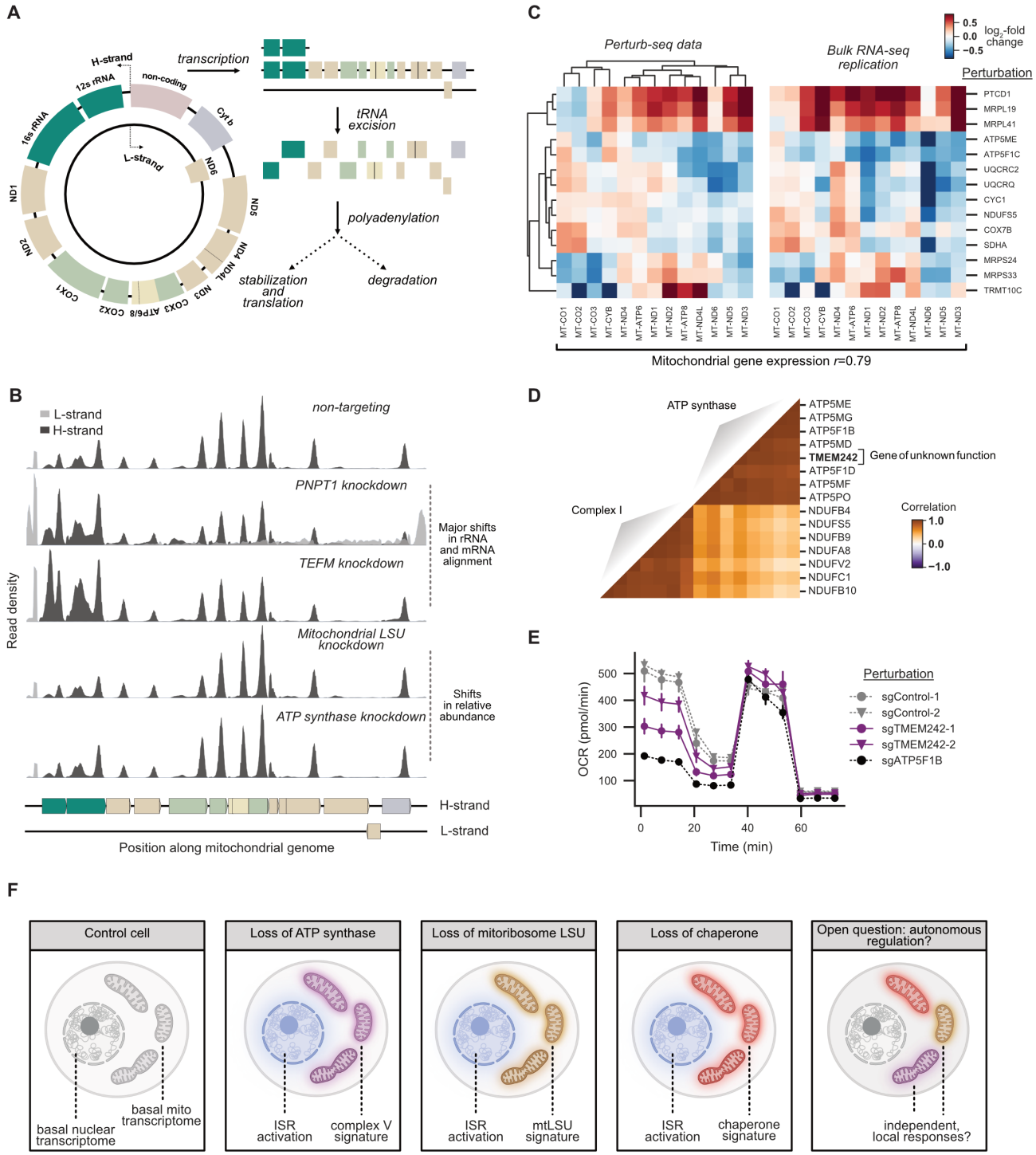

Figure 7: Investigating regulation of the mitochondrial genome in stress. (A) Schematic of the mitochondrial transcriptome. Each human cell contains many copies of the circular 16.6 kb mitochondrial genome distributed throughout the mitochondrial network. The human mitochondrial genome encodes 2 rRNAs, 22 tRNAs, and 13 protein-coding genes. Both the heavy (H) and light (L) strand of the genome are transcribed as polycistronic transcripts punctuated by tRNAs. Excision of tRNAs from transcripts generates nascent mRNA precursors (colored by complex membership). mRNA precursors can then be polyadenylated, stabilized, or

degraded. **(B)** Density of Perturb-seq reads along the mitochondrial genome from select genetic perturbations. Reads are aligned to both the H-strand (dark grey) and L-strand (light grey). For each perturbation, densities are shown relative to the maximum read count in the locus. **(C)** Comparison of mitochondrial gene expression profiles between Perturb-seq and bulk RNA-seq. Heatmap displays $\log_2$-fold changes in expression of the 13 mitochondrial encoded genes (columns) for genetic perturbations (rows) in Perturb-seq and bulk RNA-seq data collected from K562 cells. Bulk RNA-seq was conducted to analyze total RNA (including non-polyadenylated RNA), with data representing the average of biological replicates. Genetic perturbations and genes are ordered by average linkage hierarchical clustering with a Euclidean distance metric. The profiles are strongly correlated ($r=0.79$, $p<10^{-39}$). **(D)** Clustering of *TMEM242* genetic perturbation based on the mitochondrial transcriptome. Genetic perturbations to members of ATP synthase and Complex I of the respiratory chain were compared to knockdown of *TMEM242*, a mitochondrial gene of unknown function. Gene expression profiles were restricted to the 13 mitochondrially encoded genes. The heatmap displays the Pearson correlation between pseudobulk *z*-normalized gene expression profiles of mitochondrial perturbations in K562 cells. Genetic perturbations are ordered by HDBSCAN with a correlation metric. **(E)** Effect of *TMEM242* knockdown on mitochondrial respiration. A Seahorse analyzer was used to monitor oxygen consumption rate (OCR). The Mito Stress Test consists of sequential addition of oligomycin (an ATP synthase inhibitor that enables measurement of ATP-productive respiration), FCCP (an uncoupling agent that enables measurement of maximal respiratory capacity), and a mixture of rotenone and antimycin A (inhibitors of Complex I and Complex III, respectively, that enable measurement of non-mitochondrial respiration). Data is presented as average ± SEM, n=6. **(F)** Schematic diagram of mitochondrial stress response.