Responsiveness to perturbations is a hallmark of transcription factors that maintain cell identity

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Abstract

Our ability to identify the particular transcription factors that maintain cell type is limited. Identification of factors by the tissue-specificity of their expression or their participation in developmental regulation has been only modestly successful. We hypothesized that because cell type is resilient to perturbations, the transcriptional response to perturbations would identify lineage-maintaining factors. We developed Perturbation Panel Profiling (P^3) as a framework for perturbing cells in dozens of conditions and measuring gene expression responsiveness transcriptome-wide. Applying P^3 to human iPSC-derived cardiac myocytes showed that transcription factors known to function in cardiac differentiation and maintenance were among the most frequently up-regulated (most responsive). We reasoned that responsive genes may serve as lineage-maintaining factors. We identified responsive transcription factors in fibroblasts using P^3 and found that suppressing their expression led to enhanced reprogramming efficiency. We propose that responsiveness to perturbations is a key property associated with factors that maintain cellular identity.

Introduction

Cells of a particular lineage likely maintain many of their specific behavioral properties across a range of conditions. The maintenance of these properties can be the result of the activity of certain transcription factors, and the identification of these factors may be useful for manipulating cellular identity (Arendt et al., 2016). However, it has proven difficult to determine which transcription factors, of the many transcription factors associated with cellular identity, are responsible for the regulation of cellular identity.

Transcription factors can be involved in cellular identity in a number of ways. Two key functional properties of transcription factors are their ability to be *lineage-establishing* or

lineage-maintaining, meaning that they are able to control the expression of genes that will establish or maintain the characteristics of a particular cell type, respectively. A correlative property of a transcription factor is its lineage-specificity, meaning the degree to which its expression is almost entirely restricted to that one cell type and not to others. Lineage-specificity is a relatively easy property to measure, given the ubiquity of large gene expression datasets. Several recent approaches have mined these large gene expression datasets to try and identify functionally important transcription factors based on their lineage-specificity or inferred activity in particular tissues (Cahan et al., 2014; Morris et al., 2014; Rackham et al., 2016; Tomaru et al., 2014). The lineage-specificity of a transcription factor, however, is fundamentally correlative and does not *per se* yield any information about the functional properties of lineage establishment or maintenance, and so the pool of identified transcription factors may contain several non-functional candidates. Using lineage-specificity to identify causative factors can also suffer from the converse problem: it could be that there are factors that can establish or maintain cell type but are not cell-type specific in their expression or activity. Such factors would also not be easily identified by methods relying on lineage-specificity.

A related approach to identifying lineage-establishing or -maintaining factors is to focus on factors known to be involved in the developmental process responsible for differentiating cells into a particular type (Fu et al., 2013; Ieda et al., 2010; Nam et al., 2013; Olson and Srivastava, 1996; Qian et al., 2013; Takeuchi and Bruneau, 2009; Vierbuchen et al., 2010; Weintraub et al., 1989). However, in other contexts, such as transdifferentiation of cells grown in culture, there may be factors that can establish cell type via trajectories that do not mirror normal development, which could be missed by those approaches (Briggs et al., 2017; Mellis and Raj, 2015).

We hypothesized that the transcriptional response to perturbations would help identify genes whose expression is important for maintaining cell type. We envisioned two scenarios with distinct transcriptional response profiles to help maintain fate. In the first scenario, critical transcription factors maintain stable expression in the face of perturbations, that is, they are the steady, non-responsive “pillars” of expression. In the second scenario, critical transcription factors are highly reactive and frequently respond to perturbations to ensure that cells maintain their type; that is, they are hyper-responsive “springs” of expression; **Fig. 1A**. Transcriptionally profiling populations of cells subjected to a large panel of perturbations would allow us to discern whether one scenario was favored or if there was equipoise between the scenarios. Other perturbation profiling datasets have been generated, but their experimental designs have been geared towards other goals like drug target identification (Keenan et al., 2018; Niepel et al., 2019; Szalai et al., 2019).

Here, we developed an experimental and analytical pipeline, perturbation panel profiling (P^3), to measure the genome-wide transcriptional responses to a large set of perturbations in human iPSC-derived cardiac myocytes and fibroblasts. We demonstrated that well-known regulators of cardiogenesis (lineage-establishing and -maintaining transcription factors) are amongst the most responsive in their expression to a wide variety of perturbations in cardiac myocytes; i.e.,

they act as springs rather than pillars. We then applied this same logic to fibroblasts to identify highly responsive genes in that cell type. We showed that lowering the expression of fibroblast-responsive factors identified by P^3 led to an enhancement in the cells' ability to reprogram into induced pluripotent stem cells. This enhancement suggested increased cellular pliability; i.e., suppression of responsive transcription factors reduced fibroblast identity maintenance. In sum, our results demonstrate that responsiveness to perturbation may represent a common property of genes that are important for cell type maintenance.

Results

Transcription factor genes that regulate lineage identity are upregulated in cardiac myocytes after many different perturbations

To determine whether genes that are critical for cardiogenesis are more or less often responsive to perturbation than other similarly highly expressed genes, we designed an experimental and analytical workflow, P^3. P^3 consists of growing highly pure cell type populations, exposing them to a panel of dozens of different drugs in parallel, and performing RNAtag-seq for transcriptome-wide gene expression profiling in each condition. We then measure changes in gene expression across all conditions. We chose to initially apply P^3 to human iPSC-derived cardiac myocytes (iPSC-CMs) because of the ease of culture, applicability to emerging therapies, and well-established developmental trajectories. To identify potential factors in an unbiased way, we sought to perturb cells such that, in aggregate, we affected as much of the transcriptome as possible. Therefore, we sought to maximize the number of signaling cascades we perturbed, identifying 100 small molecules from the SelleckChem Bioactive library targeting any kinases or G protein-coupled receptors (GPCR) and minimizing overlap in their annotated targets (**Table S1**). We administered these drugs individually or in pairs, for a total of 75 different perturbation conditions (**Fig. S1A**).

We cultured hundreds of parallel bulk samples of iPSC-derived cardiac myocytes, cultured under standard (i.e., control, n = 63 replicate samples, spread evenly across all plates) and different (N=75) perturbed conditions (**Fig. 1B, Fig. S1A,** n= 3 replicate samples per condition). The dose for each drug was chosen to be 100 times the median IC50 values (or 10 times for each drug in a pair) culled from the literature and product materials (we determined that 10x - 100x were the appropriate dose factor empirically; data not shown; see Methods), and ultimately applied each drug at one drug-specific dose in triplicate to samples that we further analyzed with RNAtag-seq (Shishkin et al., 2015). After treating iPSC-derived cardiac myocytes with each compound or DMSO-only exposure for 4 days, we found that 63 out of 75 conditions had wells with beating cells similar in morphology to vehicle-treated cells and without evident cell death (**Fig. S1B,C**).

We next conducted transcriptome-wide gene expression profiling of the hundreds of perturbed (and control) samples of cardiac myocytes **(Fig. 1C)**. We performed RNAtag-seq in batches of 96 samples, distributing DMSO controls evenly across each batch. Gene expression profiles

across the 458 quality-controlled samples (454 excluding 4 HeLa samples used as an outgroup) clustered first by cell type, and then often by perturbation condition (**Fig. S2**). We identified hundreds to thousands (0 - 6862 genes per condition) of differentially expressed genes in most perturbed conditions relative to corresponding cell type controls (**Fig. S3A**, Methods). We sequenced our libraries to a depth at which we estimated that fully doubling the number of reads for all 458 samples would have increased the number of detected differentially expressed genes by at most only 48% for genes expressed at an average of 20 RPM or greater in controls (note that sequencing to half the depth would have reduced the number of detected differentially expressed genes by 29%; **Fig. S4**).

In order to ensure our analysis was sufficiently unibased, we verified that the majority of the coding genome was affected by a subset of our 60+ perturbations. We quantified the number of perturbations which resulted in differential expression in iPSC-derived cardiac myocytes for each gene **(Fig. 1C)**, limiting our analyses to genes which were expressed at 20 RPM or higher in control iPSC-derived cardiac myocytes. We found that 94.5% of genes expressed at 20 RPM or greater in iPSC-derived cardiac myocytes were differentially expressed in at least 1 perturbation condition relative to the cell type controls (**Fig. S3**), demonstrating that the compounds in our library were able to perturb the vast majority of the transcriptome. We focused our subsequent analyses on transcription factors, as they control the expression of genes relevant to cellular identity and homeostasis.

Next, we determined the degree to which individual transcription factors were responsive in the iPSC-derived cardiac myocyte dataset. We quantified the number of perturbations of iPSC-derived cardiac myocytes that resulted in differential expression (adj. p-value < 0.1) of a transcription factor versus controls. In addition, we wanted to ensure that responsiveness did not merely identify genes which were highly expressed or that only highly expressed genes were detectably responsive. We found that expression level did not entirely correlate with detected responsiveness: many lowly expressed transcription factors were responsive to perturbations and many highly expressed transcription factors were not responsive to perturbation. However, we observed that multiple critical cardiac transcription factors frequently responded to perturbations, including *MEF2C, NKX2-5, GATA4, HAND2, TBX5*. **(Fig. 1D-F)**. Furthermore, gene ontology analysis of all responsive genes (not just transcription factors) identified a clear over-enrichment for genes related to cardiac function (e.g., structural components such as sarcomeres, myofibrils) relative to all highly expressed genes. By contrast, non-responsive genes did not demonstrate this over-enrichment (**Fig. S6**). Prominently among the transcription factors were 6 of the 7 members of the “7F” cocktail, whose combined expression has previously been reported to induce transdifferentiation of fibroblasts into cardiac myocytes (Fu et al., 2013).

We wanted to confirm that these factors could enable such transdifferentiation in the specific fibroblast line we originally used to generate the iPSC-derived cardiac myocytes, so we overexpressed them in isogenic GM00942 fibroblasts by viral transduction. Using single-molecule RNA FISH (Raj et al., 2008), we found that around 1% of the cells expressed

the cardiac myocyte-specific marker genes *TNNT2* and *NPPA*. This efficiency is comparable to previously reported results (Fu et al., 2013; Mohamed et al., 2017). The appearance of these markers suggested that these cells were at least partially reprogrammed to a cardiac myocyte-like state (**Fig. 1H,I, S5A-E**), a finding we confirmed in two other fibroblast lines, highlighting the relevance of these factors that were re-identified by P^3 (**Fig. S5F**). In addition to their role as lineage-establishing factors, several of these factors have been demonstrated to be lineage-maintaining transcription factors, particularly *MEF2C, NKX2-5, GATA4,* and *TBX5* (Ang et al., 2016; Kathiriya et al.; Potthoff et al., 2007; Targoff et al., 2013). Taken together, these data suggest that critical cardiac lineage-establishing and -maintaining factors are not steady, non-responsive "pillars" of expression, but rather like "springs" in that they frequently respond to perturbations**.**

Responsiveness is a property of a gene's transcriptional regulation that appeared to be associated with lineage-regulating factors. Most approaches to date have, however, focused on lineage-specificity, meaning whether expression of a gene is specific to a given lineage. To compare responsiveness to lineage-specificity, we used a lineage-specificity score, which is a metric that combines expression levels and tissue-specificity as defined by various publicly available datasets. We calculated lineage-specificity scores for all genes in each tissue included in the GTEx dataset based on average expression levels (See Methods, (Cabili et al., 2011; GTEx Consortium et al., 2017)). We replaced the heart and skin GTEx samples with expression data generated from our control iPSC-derived cardiac myocytes and GM00942 fibroblasts , respectively, in order to most directly compare expression features from P^3 experiments with lineage-specificity scores. We compared the responsiveness of factors identified in our iPSC-derived cardiac myocyte and GM00942 fibroblast P^3 experiments to the iPSC-derived cardiac myocyte- or GM00942 fibroblast-specificity score, respectively **(Figures 1E,G).** We found that responsiveness and lineage-specificity score did not equate (similar results held when using all GTEx samples, **Fig. S7**). Of 14 select factors known to be relevant to cardiac myocyte biology, we found that lineage-specificity score only identified 5 of them, whereas responsiveness identified 10. However, lineage specificity was far more selective, in that of the top 24 lineage-specific factors, 5 belonged to the 14 known factors, whereas responsiveness identified 123 factors in addition to the 10 of the 14 known ones. It is possible that these additional factors represent further lineage-maintaining factors, or that they are merely other responsive factors with no functional role in lineage regulation. Further studies will be required to make such a determination (see discussion).

Knockdown of putative lineage-maintaining factors identified by P^3 enhances reprogramming

Given that P^3 identified known factors involved in maintaining cardiac myocyte identity, we reasoned that high responsiveness might also identify new, previously undescribed, factors that maintain cellular identity. One way to test this hypothesis is in reprogramming from a source to target cell type: we reasoned that the downregulation of factors that are responsive in the source cell type may interfere with source cell type maintenance, manifesting as an

enhancement of reprogramming to the target cell type. We tested this possibility by focusing on the induction of induced pluripotent stem cells from fibroblasts, i.e., fibroblast reprogramming.

To identify candidate factors that were important for maintaining fibroblast lineage identity, we performed P^3 on isogenic dermal fibroblasts (GM00942, Coriell, **Fig. 2A**). Similar to our iPSC-derived cardiac myocyte studies, we found that 62 out of 75 fibroblast culture conditions had wells with cells with fibroblast morphology and without evident cell death. We also observed similar numbers of detectably differentially expressed genes in fibroblasts as in iPSC-derived cardiac myocytes across the perturbation panel (**Fig. S3**). Responsive genes identified in our P^3 analysis again did not equate to highly expressed or tissue-specific factors in a parallel analysis to that described above for iCards-942 (**Fig. S8A,B**, **S9**). Further, Gene Ontology analysis on all responsive genes (not just transcription factors) revealed an over-enrichment for adhesion-related cellular components (e.g., cell-substrate junction) that are likely related to fibroblast function (**Fig. S6**). This enrichment mirrors the enrichment of cardiac-myocyte components amongst the set of responsive genes in iPSC-derived cardiac myocytes.

To test whether the set of responsive transcription factor genes was enriched for factors whose downregulation could enhance reprogramming efficiency, we first chose 16 transcription factors that were often responsive in fibroblasts in our P^3 experiments, regardless of their lineage-specificity to fibroblasts. We considered any transcription factor genes that were 1) up-regulated in at least 5 conditions in GM00942, 2) up-regulated in at least 50% more conditions than they were down-regulated in GM00942, 3) not also frequently up-regulated in iPSC-derived cardiac myocytes, 5) expressed at >50 TPM in GM00942 controls, 6) not commonly studied in the context of fibroblast development based on literature review, 7) not commonly considered a member of a stress response or apoptosis pathway based on literature review, and 8) with at least 3 quality-controlled targeting shRNA clones available through our university core service lab's Human TRC 2.0 lentivirus library (See Methods). Out of the set of 66 highly responsive factors identified by P^3 in GM00942, 41 met all of the above criteria, of which we tested 16 for practical experimental scale considerations. We selected these 16 factors for validation based on them having either the most conditions in which it was up-regulated or the highest ratio of conditions in which it was up- vs. down-regulated. The human fibroblast line we used to test for reprogramming efficiency changes was the hiF-T line (Cacchiarelli et al., 2015), which has an integrated cassette encoding the Yamanaka factors (*OCT4, SOX2, KLF4, c-MYC*; OSKM (Takahashi and Yamanaka, 2006)) under doxycycline-inducible control. We used shRNAs to knock down the expression of these 16 factors one at a time in hiF-T cells prior to the induction of OSKM, then induced OSKM to check for differences in reprogrammability. (**Fig. 2C, S9**, **Table S2**). Of these 16 transcription factors, we found that 8 increased the number of Alkaline Phosphatase-positive colonies after 3 weeks of OSKM expression (**Fig. 2B,D,E; S9 and S10**). We further confirmed reprogramming by staining for Tra-1-60, a surface marker of induced pluripotent stem cells (Chan et al., 2009). We found that colonies positive for Alkaline Phosphatase activity also express Tra-1-60 (**Fig. 2F**). Therefore, transcription factor gene expression responsiveness in fibroblasts identified genes important in maintaining fibroblast identity.

Discussion

The ubiquity of transcriptomic measurements has enabled us to profile the expression levels of genes across virtually all tissue types and, soon enough, all cell types. However, while these expression profiles may reveal which factors are associated with a particular cell type, such factors may or may not play a functional role in maintaining said cell type. We have found that high responsiveness of expression to perturbations may be a way to identify factors that play this functional role; i.e., these factors are “springs” that pull cells back to their identity, rather than “pillars” that remain constant in the face of perturbations.

The most prevalent way to identify candidate factors for maintaining cell type is to analyze compendiums of transcriptional profiles across various tissue types and look for genes whose expression or activity is tissue specific. In principle, responsiveness could have identified a proper subset of the tissue-specific genes, a superset of the tissue-specific genes, a wholly distinct set of genes, or a partially overlapping subset. We found that responsiveness seemed to identify a superset of the tissue-specific genes, meaning that there were many genes whose expression was responsive but not particularly tissue specific. When we explicitly tested whether these responsive-but-not-tissue-specific genes could affect maintenance, we found that, for fully half the factors we tested, suppression of the factor led to an increase in reprogramming efficiency. This validation rate suggests that these responsive factors are indeed important for cell type maintenance and not merely falsely identified by P^3. The fact that these non-tissue-specific factors are important for maintenance suggests that cell type maintenance may involve factors that act as general barriers to cell type transformations in addition to factors specific to particular types. (Although it is possible that such factors may only act as barriers in particular cell types.) Our results indicate that there are many more cell type maintenance factors than tissue-specificity alone would suggest.

We posit there may be at least two reasons why lineage-maintaining factors are highly responsive to perturbation. One possibility is that lineage-maintaining factors are simply downstream of more signaling pathways than other transcription factors in the cell type that they maintain, hence their increased frequency of responsiveness to a panel of perturbations. Given that the responsiveness of genes was different in the two cell types that we performed P^3 in, such a rationale would require the often invoked “cell-type specific regulatory differences”, however. Another possibility is that the upregulation of these genes is part of a feedback mechanism that mediates a homeostatic response to a perturbation. Such a response may be particular to specific signals, or may be part of a more general response to a perturbative change in some aspect of the physiology or regulation of a cell’s identity. Given that the genes identified by P^3 were responsive to several perturbations, we suspect the latter. Another interesting question about responsiveness is whether the responses are transient or sustained. The presence of feedback loops or other homeostatic mechanisms may temper the signal over time, leading to transient responses, whereas the lack of such mechanisms may yield sustained

responses. Temporal measurements may help distinguish between transient and stable responses.

It is important to distinguish between lineage-establishing factors versus lineage-maintaining factors. Although some lineage-establishing genes are also lineage-maintaining, such as *GATA4* and *TBX5* in cardiac myocytes or *OCT4* in pluripotent stem cells (Ang et al., 2016; Kathiriya et al.; Shi and Jin, 2010), the converse need not be frequently true. That is, under the interpretation that responsiveness is a hallmark of lineage maintenance, then one could assume that responsive factors would not necessarily also help establish a new lineage altogether. Consistent with this logic, we found that the transdifferentiation of fibroblasts to cardiac myocytes was not enhanced by adding responsive factors to the existing seven factor (7F) cocktail (**Fig. S12**). (Technically, however, it is far more difficult to evaluate the effects of overexpressing large combinations of factors (Addis et al., 2013; Guo and Morris, 2017; Nam et al., 2014; Qian et al., 2013; Wang et al., 2015)). Other approaches may be required to solve that complementary question.

An alternative approach that has been used to identify lineage-maintaining factors is genetic screens (Cacchiarelli et al., 2015; Liu et al., 2018; Parekh et al., 2018; Zhou et al., 2017). Cacchiarelli et al. used pooled shRNA screens targeted towards a set of epigenetic factors to identify a number of such maintenance factors. It is difficult, however, to perform such screens in a genome-wide fashion owing to technical considerations. We envision that P^3 may serve as a useful complement to such screens by providing a means by which one could identify on the order of a hundred candidate factors, which could subsequently be tested in a pooled screen. Such a combined approach may allow for rapid and unbiased identification of identity-maintaining factors.

Materials and Methods

Cell culture

Unless otherwise noted, all cell culture incubations below were performed at 37°C, 5% CO_2. We tested intermittently for mycoplasma contamination.

GM00942 human dermal fibroblast culture

We cultured GM00942 human dermal fibroblasts (Coriell, GM00942; XX donor, normal-appearing tissue) according to the distributor's instructions, on tissue culture-treated dishes in E-MEM (QBI 112-018-101) + 10% FBS (Life Technologies 16000044, lot 1802004) + Pen/Strep.

GM11169 human cardiac fibroblast culture

We cultured GM11169 human cardiac fibroblasts (Coriell, GM11169; XX donor) on tissue culture-treated dishes in DMEM w/Glutamax + 9% FBS (Life Technologies 16000044) + P/S.

HEK293FT culture

We expanded HEK293FT cells in DMEM w/Glutamax + 9% FBS + P/S.

hiF-T culture

We cultured hiF-T cells as previously described prior to hiF-T-iPSC reprogramming experiments(Cacchiarelli et al., 2015). Briefly, we expanded hiF-T cells in growth medium on TC plastic dishes coated with Attachment Factor (Fisher S006100), and split cells 1:3 when they reached 60-70% confluency. hiF-T growth medium (GM) is DMEM/F-12 w/ Glutamax (Life Tech. 10565018) + 10% ES-FBS (Life Tech. 16141079) + 1x 2-Mercaptoethanol (Life Tech. 21985023) + 1x NEAA (Invitrogen 11140050) + P/S + 0.5µg/mL Puromycin + 16ng/mL rhFGF-basic (Promega G5071).

Immortalized human cardiac fibroblast (immHCF) culture

We cultured immortalized human cardiac fibroblasts (HCFs) as previously described (Mohamed et al., 2017). We received HCFs from Deepak Srivastava (Gladstone Institutes/UCSF). In brief, we expanded HCFs on gelatin-coated (Millipore ES-006-B) tissue culture dishes in iCM medium (per 500mL: 350mL DMEM w/Glutamax + 85mL Medium 199 + 50mL FBS + 5mL Non-essential amino acids + 10mL Pen/strep).

Platinum-A (Plat-A) retroviral packaging cell line culture

We cultured Platinum-A cells (Cell Biolabs RV-102) according to the manufacturer's instructions. We expanded these cells in DMEM w/Glutamax + 9% FBS + P/S + 10ug/mL Blasticidin + 1µg/mL Puromycin.

Cellular reprogramming

Derivation of cardiac myocytes (iPSC-CMs) from iPS-GM942-SeV3 iPSCs

We differentiated cardiomyocytes from iPS-GM942-SeV3 cells to create iPS-GM942-SeV3-CMs, as previously described(Laflamme et al., 2007; Palpant et al., 2015; Shanmughapriya et al., 2018; Zhu et al., 2010, 2011). Throughout this manuscript we refer to iPS-GM942-SeV3-CMs as 'iPSC-derived cardiac myocytes' or 'iPSC-CMs'. Briefly:

Seeding of iPS-GM942-SeV3

We thawed iPS-GM942-SeV3 and grew them in feeder-free conditions on Geltrex-coated (ThermoFisher, cat. A1413301) dishes in StemMACS iPS Brew-XF (Miltenyi, cat. 130-104-368) for 4-5 days, until ~75% confluency. Then, we split and seeded iPS-GM942-SeV3 into Geltrex-coated 12-well plates at a density of 3 x 10^5 cells per well in iPS-Brew + 2µM Thiazovivin (Sigma, cat. SML1045-5MG). After 24 hours, we changed the culture medium to iPS-Brew + 1µM Chiron 99021 (Cayman Chemical, cat. 13122) and then incubated the cells for an additional 24 hours.

Differentiation to iPS-GM942-SeV3-CMs (a.k.a. iPSC-CMs)

Starting on Day 0, we incubated iPS-GM942-SeV3 for 18 hours in RPMI (Life Technologies, cat. 11875-119) + 100ng/ml Activin A (R&D systems, cat. 338-AC- 010) + 2% B-27 (minus insulin; Life Technologies, cat. 17504-044). Next, on Day 1 we changed the medium to RPMI + 2% B-27 (minus insulin) + 5ng/ml BMP4 (Peprotech, cat. AF-120-05ET) + 1uM Chiron 99021 and incubated these cells for 48 hours. On Day 3 we changed the medium to RPMI + 2% B-27 (minus insulin) + 1uM Xav 939 (Tocris Bioscience, cat. 3748) and incubated the cells for 48 hours. On Day 5 we changed the medium to RPMI + 2% B-27 (minus insulin) and incubated the cells for 72 hours. From Day 8 - 12 we changed the medium to RPMI + 2% B-27 (including insulin) + 1% pen/strep, and replaced the medium every other day.

Glucose-free medium selection steps for cardiac myocytes

In order to enrich the culture for cardiac myocytes, we subjected these cells to two low glucose selection steps. On Day 12 we started the first selection step by replacing the medium with RPMI glucose free (ThermoFisher, cat. 11879020) + 2% B-27 (including insulin) + 1% Pen-Strep and incubated for 72 hours. On Day 15 we replated the cells onto Geltrex-coated dishes at 6.3e5 cells/cm^2 in RPMI + 20% FBS (Seradigm, lot 050B14) + 1uM Thiazovivin and incubated them for 24 hours. On Day 16 we changed the medium to RPMI + 2% B-27 (including insulin) and incubated them for 48 hours to recover. On Day 18 we started the second selection step, again by replacing the medium with RPMI glucose free (ThermoFisher, cat. 11879020) + 2% B-27 (including insulin) + 1% Pen-Strep and incubated for 72 hours.

Replating iPS-GM942-SeV3-CMs into 96-well plates

On Day 21 of differentiation, we passaged the iPS-GM942-SeV3-CMs into Geltrex-coated 96-well plates at a density of 1e5 cells per well in RPMI + 20% FBS + 1μM thiazovivin and incubated overnight. On Day 22 we changed the medium to RPMI + 2% B-27 (with insulin) + antibiotics and cultured for an additional 48 hours to allow the plated cultures to start contracting again. By Day 23 or 24 we expected to observe recovery of contractile activity among a majority of the cardiac myocytes in all of the wells. If we did not observe beating activity we changed the RPMI + 2% B-27 (with insulin) + pen/strep medium on Day 24, incubated for another 48 hours, and checked again for contractile activity on Day 26. If the cardiac myocytes did not regain contractile activity by Day 26, we did not proceed to perturbation culture.

<u>Perturbation culture</u>

GM00942 fibroblasts

For GM00942 fibroblasts, we called the day on which they were seeded in 96-well plates Day -2 of perturbation. We split 95% confluent 10cm tissue culture-treated dishes of cells into 96-well plates at a density of roughly ~1.5e4 cells per well in EMEM + 10% FBS + Pen/strep and incubated for 48 hours. On Day 0 we replaced the medium (250 μL) in each well and added 0.7 μL of drug stock in DMSO (see **Table S1**) or of DMSO (for control cultures). This kept total DMSO concentration of the perturbation culture medium below 0.3% for all conditions. We incubated cells for 48 hours, and then on Day 2 replaced medium and re-adding a fresh dose of drug stock at the same volumes as Day 0. We incubated cells for a further 48 hours before taking images of each well (below) and extracting RNA (below) on Day 4.

iPSC-derived cardiac myocytes

For iPSC-CMs, we called the first day on which we observed beating activity in the majority of wells of each 96-well plate Day 0 of perturbation. On Day 0 we replaced the medium with 250μL RPMI + 2% B-27 (with insulin) + pen/strep and added 0.7μL of drug stock in DMSO (See **Table S1**) or of DMSO (for control cultures), again keeping total DMSO concentration of perturbation culture medium below 0.3% for all conditions. We incubated cells for 48 hours, and then on Day 2 replaced medium and re-adding a fresh dose of drug stock at the same volumes as Day 0. We incubated cells for a further 48 hours before taking videos of each well (below) and extracting RNA (below) on Day 4.

Transdifferentiation of fibroblasts to induced cardiac myocyte-like cells

We performed transdifferentiation of immortalized HCFs (immHCF), GM11169 fibroblasts, and GM00942 fibroblasts to induced cardiac myocyte-like cells as previously described(Mohamed et al., 2017). Briefly, on day -3 we plated 10^4 fibroblasts per well in 12-well culture vessels in iCM medium and Plat-A cells in 10cm dishes (4e6 cells per dish) in DMEM w/Glutamax + 9% FBS without any antibiotics. On day -2 we transfected each dish of Plat-A cells with 10ug of one indicated pMXs expression plasmid in 500uL Optimem + 35uL Fugene HD. On day 0, we collected viral supernatants and pooled them as needed for replicate conditions, filtered them through 0.45µm filter units, and transduced fibroblasts. For transductions we used 6µg/mL polybrene, a 30min 930 x g spin, and overnight incubation at 37C. On day 1 we replaced transduction medium with iCM medium. On day 4 we replaced iCM medium with 75% iCM medium/25% Reprogramming medium (RPMI 1640 + B-27 + P/S), on day 7 with 50% iCM medium/50% Reprogramming medium, on day 11 with 25% iCM medium/75% Reprogramming medium, and on day 14 with Reprogramming medium alone. We then changed reprogramming medium daily until analysis on the indicated day per experiment, usually day 24. On day 24, we fixed cells in two formats for analysis. Some 12-well TC plastic wells were fixed in place using 3.7% formaldehyde and permeabilized at least overnight with 70% Ethanol in 4C, while others were dissociated with Accutase and transferred to Concanavalin-coated (Sigma C0412) 8-well Lab-Tek chambers. After 90-120 minutes, transferred samples were fixed in these 8-well chambers using 3.7% formaldehyde and permeabilized at least overnight with 70% ethanol in 4C. Samples in 12-well wells were processed for FISH imaging by excising them from their 12-well plate after fixation with a heated 20mm cork borer and processing them for FISH or immunofluorescence as described below.

Cloning of transcription factor genes and TurboGFP into pMXs

In order to drive overexpression of perturbable transcription factor genes and TurboGFP, we cloned cDNA for genes of interest into pMXs-gw (Addgene 18656; a gift from Shinya Yamanaka) using BP and LR Clonase II (Invitrogen). We amplified cDNA of targets of interest using attB-target-specific primers (**Table S3**). We used standard tools to verify sequence identity of the plasmid backbone and gene insert, such as restriction digestion and Sanger sequencing. We amplified attB-TurboGFP off of the SHC003 plasmid (Sigma SHC003).

Titering of pMXs retroviral vectors

Since our expression vectors do not contain selectable or fluorescent markers and pMXs retroviral vectors only transduce dividing cells, we indirectly titered each experimental replicate's batch of virus by co-transducing parallel samples of HCFs with pMXs-DsRed Express (Addgene 22724; a gift from Shinya Yamanaka) and pMXs-TurboGFP (see "Cloning" above) produced using the same batch of Plat-A cells under the same conditions. In order to estimate the fraction of cells that are infected at least once per factor, we considered the infection rates of these fluorescent pMXs vectors. By comparing the fraction that are co-infected with both against the fraction that are infected with each factor at all, we can infer the fraction of cells dividing in the population during the transduction period and the fraction of those cells that receive at least one copy of any individual expression vector. We make the simplifying assumption that among dividing cells infection events are independent of each other. Therefore, the ratio of the fraction

that are DsRed+ and GFP+ to the fraction that are DsRed+ (or GFP+) is approximately the square of the transduction rate for any individual virus. E.g., for 30% of cells being DsRed+ and 24.3% being DsRed+ and GFP+, 24.3/30 = 0.81, which gives 90% transduction rate for each individual virus. We used two-color flow cytometry (GUAVA) to assess DsRed+, GFP+, and DsRed+ GFP+ fractions per sample.

shRNA-mediated knockdown of transcription factors in hiF-T cells

We conducted knockdown of individual transcription factors using shRNAs essentially as previously described.(Cacchiarelli et al., 2015) In brief, we acquired cloned pLKO.1, pLKO.1-shRNA, and pLKO.1-TurboGFP plasmids from the University of Pennsylvania High-Throughput Screening Core (**Table S2**). We verified shRNA and backbone sequence with Sanger sequencing. We packaged shRNA lentivirus using pMD2.G (Addgene 12259; a gift from Didier Trono) and psPAX2 (Addgene 12260; a gift from Didier Trono) in HEK293FT cells, and filtered viral supernatant through 0.22μm filter units prior to infecting hiF-T cells. We infected hiF-T cells at an MOI of approximately 1 (for a transduction efficiency of ~70%) with 4μg/mL polybrene and 30 min 930 x g centrifugation. Since hiF-T cells are already Puromycin-resistant, we were unable to perform an antibiotic selection step after infection with these pLKO.1-puro-based shRNA plasmids.

Verification of knockdown efficiency following shRNA transduction

We performed RT-qPCR on RNA extracted from samples of the hiF-T cells that we used in reprogramming experiments. We used Superscript III Reverse Transcriptase for first-strand cDNA synthesis and Power SYBR qPCR Master Mix with gene-specific primer pairs for qPCR on an Applied Biosystems 7300 system. We performed all statistical analysis using custom scripts in R (see “Code accessibility” below for all scripts) and calculated knockdown efficiency using the ΔΔCt method.

hiF-T reprogramming to pluripotency

We performed hiF-T reprogramming experiments as previously described. Briefly, after shRNA transduction on day -7, we expanded cells in hiF-T GM without puromycin for one week. On Day -1 we seeded CF-1 Irradiated MEFs on uncoated 24-well plates (Corning) at a density of 2.5 x 10^5 cells per well in hiF-T GM without puro. On Day 0, we seeded 10^4 hiF-T cells per 24-well plate well. On Day 1 we began Yamanaka factor induction by switching media to hiF-T GM with 2μg/mL doxycycline and without puromycin. On Day 3 we switched media to KSR Medium (KSRM): DMEM/F-12 w/ Glutamax (Life Tech. 10565018) + 20% Knockout Serum Replacement (Life Tech. 10828010) + 1x 2-Mercaptoethanol (Life Tech. 21985023) + 1x NEAA (Invitrogen 11140050) + P/S + 8ng/mL rhFGF-basic + 2μg/mL Doxycyclin. We changed KSRM daily, and analyzed cells on day 21. We performed a total of 3 biological replicates (i.e., different vials of hiF-T cells expanded and reprogrammed on different days with different batches of media), each with technical triplicates per shRNA condition. All biological and technical replicates of all reprogramming experiments we conducted are presented in this manuscript.

High-throughput RNA extraction

We used RNaqueous-96 kits (Ambion AM1920) for RNA extraction without the optional DNase step, according to manufacturer's instructions.

RNAtag sequencing

We conducted highly parallelized bulk RNA sequencing with RNAtag-seq as previously described, using all components and steps in the published protocol (Shishkin et al., 2015). We ordered the specified 32 barcoded DNA oligos for RNAtags from Biosearch Technologies and indexed primers for library amplification and reverse transcription from IDT. We sequenced all RNAtag-seq libraries in batches of 96 samples on an Illumina NextSeq 550 using 75 cycle high-output kits (Illumina 20024906).

RNAtag-seq data processing

We demultiplexed RNAtag-seq reads using custom scripts, courtesy of Edward Wallace (Wallace and Beggs, 2017). We aligned RNAtag-seq reads to the human genome (hg19) with STAR v2.5.2a and counted uniquely mapping reads with HTSeq v0.6.1 (Shaffer et al., 2017). We performed all downstream analysis in R v3.6.1 using packages yaml_2.2.0, DESeq2_1.24.0, SummarizedExperiment_1.14.0, DelayedArray_0.10.0, BiocParallel_1.18.0, matrixStats_0.54.0, Biobase_2.44.0, GenomicRanges_1.36.0, GenomeInfoDb_1.20.0, IRanges_2.18.1, S4Vectors_0.22.0, BiocGenerics_0.30.0, e1071_1.7-2, magrittr_1.5, ggrepel_0.8.1, ggplot2_3.2.0, tibble_2.1.3, tidyr_0.8.3, and dplyr_0.8.3, clusterProfiler_3.12.0, org.Hs.eg.db_3.8.2, and their associated dependencies.

Gene expression perturbation-responsiveness

As a measure of gene expression perturbation-responsiveness we used the count of the number of conditions in which a gene was differentially expressed relative to cell type DMSO controls. For most analyses we used any change with a DESeq2 adjusted p-value less than 0.1, but also conducted analyses with additional filters, such as minimum absolute values of log2FoldChange. Filter criteria are explicitly noted in figure legends for each analysis. We also considered other measures of perturbation-responsiveness, as well, which are not explored in the manuscript above, details of which are available upon request.

Gene expression tissue-specificity analysis

We calculated Jensen-Shannon divergence-based scores for each gene as previously described (Cabili et al., 2011) in each tissue or cell type in the GTEx V7 RNA-seq dataset (GTEx Consortium et al., 2017). Briefly, we analyzed all V7 GTEx RNA-seq samples with: RIN (RNA integrity score) ≥ 7, mapping rate ≥ 50%, and 1 million or more mapped reads. With these filters, 3623 samples remained in the processed GTEx dataset. We then calculated JS_{sp} values for each gene in each tissue type (SMTS identifier) based on average expression levels per tissue type in transcripts per million (TPM), as described in Cabili et al., 2011. For the analysis presented in main text figures, we removed GTEx Skin and Heart from the dataset and added GM00942 fibroblast and iCard-942 cardiac myocyte control average expression expression values. We present specificity score calculations with and without removal of GTEx Skin and

Heart and with and without addition of GM00942 fibroblast and iCard-942 cardiac myocyte data in the supplement.

Gene set enrichment analysis

We performed gene set over-enrichment analysis on highly expressed genes (mean RPM > 20) for each cell type using the clusterProfiler v3.12.0 package in R v3.6.1 (Yu et al., 2012). We compared iCard-responsive highly expressed genes (up-regulated in 4 or more conditions and mean RPM > 20 in control iCard-942) against the entire set of genes expressed > 20 RPM in iCard-942 controls. We compared iCard-unresponsive genes (up-regulated in 0 or 1 conditions and mean RPM > 20 in control iCard-942) against the entire set of genes expressed > 20 RPM in iCard-942 controls. We compared fibroblast-responsive highly expressed genes (up-regulated in 4 or more conditions and mean RPM > 20 in control GM00942 fibroblasts) against the entire set of genes expressed > 20 RPM in GM00942 controls. We compared fibroblast-unresponsive genes (up-regulated in 0 or 1 conditions and mean RPM > 20 in control GM00942) against the entire set of genes expressed > 20 RPM in GM00942 controls.

Prioritization of highly responsive genes for use in reprogramming experiments

For iPSC reprogramming experiments we considered transcription factor genes (Vaquerizas et al., 2009) that were 1) up-regulated in at least 5 conditions in GM00942, 2) up-regulated in at least 50% more conditions than they were down-regulated in GM00942, 3) not also frequently up-regulated in iCard-942, 5) expressed at >50 TPM in GM00942 controls, 6) not commonly studied in the context of fibroblast development based on literature review , 7) not commonly considered a member of a stress response or apoptosis pathway based on literature review, and 8) with at least 3 quality-controlled targeting shRNA clones available through our university core service lab's Human TRC 2.0 lentivirus library. Ultimately we tested knockdown of the genes 'SKIL', 'YBX3', 'TSC22D1', 'CERS2', 'KLF13', 'TBX3', 'ID1', 'ATOH8', 'ZNF652', 'NFATC4', 'ZBTB38', 'LARP1', 'CEBPB', 'ID3', 'PRRX2', and 'RUNX1'.

For cardiac transdifferentiation experiments we considered transcription factor genes (Vaquerizas et al., 2009) that were 1) up-regulated in at least 4 conditions in iCard-942, 2) up-regulated in at least 50% more conditions than they were down-regulated in iCard-942, 3) not also frequently up-regulated in GM00942, 5) expressed at >50 TPM in iCard-942 controls, 6) not commonly studied in the context of fibroblast development based on literature review, and 7) not commonly considered a member of a stress response or apoptosis pathway based on literature review. Ultimately we tested overexpression of the genes 'SP3', 'ZBTB10', 'ZBTB44', 'SSH2', 'NFIA', 'ZNF652', and 'ZFP91'.

Live cell Tra-1-60 imaging

In a pilot reprogramming experiment without shRNA transduction, we conducted live-cell staining of hiF-T-iPSC colonies Tra-1-60 with TRA-1-60 Alexa Fluor™ 488 Conjugate Kit for Live Cell Imaging (Life Tech. A25618) according to the manufacturer's instructions.

Alkaline phosphatase staining with colorimetry

We used the Vector Red Substrate kit (Vector Labs SK-5100) to stain hiF-T-iPSC colonies after fixation on day 21 of reprogramming experiments. We fixed wells in 24-well format using 3.7% formaldehyde for 3 minutes, and followed the manufacturer's instructions.

Immunofluorescence

We performed immunofluorescence for several markers. For Tra-1-60 immunofluorescence of hiF-T-iPSC samples that had already been stained with Vector Red, we blocked and permeabilized in 5% BSA + 0.1% Triton X-100 in PBS at room temperature for 30 min. Then we washed samples in PBS and used Stemgent 09-0068 at 1:200 in 5% BSA + 0.1% Triton X-100 for 2 hours at room temp. We washed samples in PBS and stained with DAPI prior to imaging. For cardiac troponin immunofluorescence of iCard-942 and transdifferentiated samples, we fixed samples in 3.7% formaldehyde for 10 min at room temp, washed in PBS, and permeabilized with 70% ethanol overnight at 4C. Independent of smFISH or after the smFISH protocol, we performed immunofluorescence with Abcam ab45932 primary (1:200) with goat anti-rabbit-Alexa 594 (1:200) secondary and Fisher MA5-12960 primary (1:200) with donkey anti-mouse-Alexa 488 (1:200) secondary. We used samples in 3% BSA + 0.1% Tween 20 for blocking/binding buffer. Primary antibody incubations of 1 hour and secondary incubations of 30 min, both at room temperature. Samples were washed with PBS and stained with DAPI prior to imaging.

Single-molecule RNA FISH

GAPDH probes were used as previously described (Padovan-Merhar et al., 2015). We incubated our cells overnight at 37°C in hybridization buffer (10% dextran sulfate, 2× SSC, 10% formamide) with standard concentrations of RNA FISH probes (**Table S3**) (Raj et al., 2008). The following morning, we performed two washes in wash buffer (2X SSC, 10% formamide), each consisting of a 30-min incubation at 37°C. After the second wash, we rinsed once with 2X SCC/DAPI and mounted the sample for imaging in and 2X SSC (Raj et al., 2008). We performed RNA FISH on cell culture samples grown on a Lab-Tek chambered coverglass using 50 µL of hybridization solution spread into a thin layer with a coverslip and placed in a parafilm-covered culture dish with a moistened Kimwipe to prevent excessive evaporation.
Imaging. We imaged each sample on a Nikon Ti-E inverted fluorescence microscope using a 60X Plan-Apo objective and a Hamamatsu ORCA Flash 4.0 camera. For 60X imaging of complete cells, we acquired z-stacks (0.3 µm spacing between slices). For 60X imaging of a large field of cells with one plane each, we used Nikon Elements tiled image acquisition with perfect focus. All images of stained cells were in different fluorescence channels using filter sets for DAPI, Atto 488, Cy3, Alexa 594, and Atto 647N. The filter sets we used were 31000v2 (Chroma), 41028 (Chroma), SP102v1 (Chroma),17 SP104v2 (Chroma) and SP105 (Chroma) for DAPI, Atto 488, Cy3, Atto 647N/Cy5 and Atto 700, respectively. A custom filter set was used for Alexa 594/CalFluor610 (Omega). We tuned the exposure times depending on the dyes used: 400 ms for probes in Cy3 and Alexa 594, 500 ms seconds for each probe in Atto 647N, and 50 ms for DAPI probes. We also acquired images in the Atto 488 channel with a 400 ms exposure as a marker of autofluorescence.

Image Processing
smFISH analysis of image scans and stacks was done as previously described using rajlabimagetools changeset 775fd10 (https://bitbucket.org/arjunrajlaboratory/rajlabimagetools/wiki/Home) in MATLAB v2019a, and is compatible with rajlabimagetools changeset a2c6ac5 (https://github.com/arjunrajlaboratory/rajlabimagetools/) in MATLAB v2017a (Raj et al., 2008).

Reproducible analysis
For all raw imaging and qPCR data, please see: https://www.dropbox.com/sh/2ny7k6c4zy6zdsh/AADLNoom3YOw0Ps0B8w509R7a?dl=0. For all raw sequencing data, please see: https://www.dropbox.com/sh/ss7eovrclone69a/AADAkowTjNZ2_FwxZSXey9rCa?dl=0. For a fully reproducible processing pipeline, including raw qPCR data, from all raw data types to the graphs and images included in this paper, please see: https://www.dropbox.com/sh/3iqo4mjx4le0a8c/AACz1J2J6Vwuq_3mLNInkYU-a?dl=0. For details, please see the readme files included in each folder.

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Author Contributions

AR and IAM conceived of P^3, and IAM, RJ, and AR conceived of the larger project with applications to cardiac transdifferentiation and reprogramming; IAM designed, conducted, and analyzed all experiments with supervision by AR and RJ, with the exception of iCard-942 differentiation performed by HIE, RT, and WY. OS contributed to the design of some transdifferentiation experiments. WY and RT contributed to analysis of reprogramming experiments. YG contributed to some transdifferentiation experiments, analysis, and figure design. MCD, PPS, and RALS contributed to some transdifferentiation experiments. IAM designed all analyses and wrote all software, with some assistance from LEB, with supervision by AR and RJ. IAM, RJ, and AR wrote the paper.

References

Addis, R.C., Ifkovits, J.L., Pinto, F., Kellam, L.D., Esteso, P., Rentschler, S., Christoforou, N., Epstein, J.A., and Gearhart, J.D. (2013). Optimization of direct fibroblast reprogramming to cardiomyocytes using calcium activity as a functional measure of success. J. Mol. Cell. Cardiol. *60*, 97–106.

Ang, Y.-S., Rivas, R.N., Ribeiro, A.J.S., Srivas, R., Rivera, J., Stone, N.R., Pratt, K., Mohamed, T.M.A., Fu, J.-D., Spencer, C.I., et al. (2016). Disease Model of GATA4 Mutation Reveals Transcription Factor Cooperativity in Human Cardiogenesis. Cell *167*, 1734–1749.e22.

Arendt, D., Musser, J.M., Baker, C.V.H., Bergman, A., Cepko, C., Erwin, D.H., Pavlicev, M., Schlosser, G., Widder, S., Laubichler, M.D., et al. (2016). The origin and evolution of cell types. Nat. Rev. Genet.

Briggs, J.A., Li, V.C., Lee, S., Woolf, C.J., Klein, A., and Kirschner, M.W. (2017). Mouse embryonic stem cells can differentiate via multiple paths to the same state.

Cabili, M.N., Trapnell, C., Goff, L., Koziol, M., Tazon-Vega, B., Regev, A., and Rinn, J.L. (2011). Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. Genes Dev. *25*, 1915–1927.

Cacchiarelli, D., Trapnell, C., Ziller, M.J., Soumillon, M., Cesana, M., Karnik, R., Donaghey, J., Smith, Z.D., Ratanasirintrawoot, S., Zhang, X., et al. (2015). Integrative Analyses of Human Reprogramming Reveal Dynamic Nature of Induced Pluripotency. Cell *162*, 412–424.

Cahan, P., Li, H., Morris, S.A., Lummertz da Rocha, E., Daley, G.Q., and Collins, J.J. (2014). CellNet: network biology applied to stem cell engineering. Cell *158*, 903–915.

Chan, E.M., Ratanasirintrawoot, S., Park, I.-H., Manos, P.D., Loh, Y.-H., Huo, H., Miller, J.D., Hartung, O., Rho, J., Ince, T.A., et al. (2009). Live cell imaging distinguishes bona fide human iPS cells from partially reprogrammed cells. Nat. Biotechnol. *27*, 1033–1037.

Fu, J.-D., Stone, N.R., Liu, L., Spencer, C.I., Qian, L., Hayashi, Y., Delgado-Olguin, P., Ding, S., Bruneau, B.G., and Srivastava, D. (2013). Direct reprogramming of human fibroblasts toward a cardiomyocyte-like state. Stem Cell Reports *1*, 235–247.

GTEx Consortium, Laboratory, Data Analysis &Coordinating Center (LDACC)—Analysis Working Group, Statistical Methods groups—Analysis Working Group, Enhancing GTEx (eGTEx) groups, NIH Common Fund, NIH/NCI, NIH/NHGRI, NIH/NIMH, NIH/NIDA, Biospecimen Collection Source Site—NDRI, et al. (2017). Genetic effects on gene expression across human tissues. Nature *550*, 204–213.

Guo, C., and Morris, S.A. (2017). Engineering cell identity: establishing new gene regulatory and chromatin landscapes. Curr. Opin. Genet. Dev. *46*, 50–57.

Ieda, M., Fu, J.-D., Delgado-Olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B.G., and Srivastava, D. (2010). Direct reprogramming of fibroblasts into functional cardiomyocytes by

defined factors. Cell *142*, 375–386.

Kathiriya, I.S., Rao, K.S., Iacono, G., Patrick Devine, W., P., A., Hota, S.K., Lai, M.H., Garay, B.I., Thomas, R., Gong, Z., et al. 1 Modeling human TBX5 haploinsufficiency predicts regulatory networks for congenital heart 2 disease.

Keenan, A.B., Jenkins, S.L., Jagodnik, K.M., Koplev, S., He, E., Torre, D., Wang, Z., Dohlman, A.B., Silverstein, M.C., Lachmann, A., et al. (2018). The Library of Integrated Network-Based Cellular Signatures NIH Program: System-Level Cataloging of Human Cells Response to Perturbations. Cell Syst *6*, 13–24.

Laflamme, M.A., Chen, K.Y., Naumova, A.V., Muskheli, V., Fugate, J.A., Dupras, S.K., Reinecke, H., Xu, C., Hassanipour, M., Police, S., et al. (2007). Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat. Biotechnol. *25*, 1015–1024.

Liu, Y., Yu, C., Daley, T.P., Wang, F., Cao, W.S., Bhate, S., Lin, X., Still, C., Liu, H., Zhao, D., et al. (2018). CRISPR Activation Screens Systematically Identify Factors that Drive Neuronal Fate and Reprogramming. Cell Stem Cell *0*.

Mellis, I.A., and Raj, A. (2015). Half dozen of one, six billion of the other: What can small- and large-scale molecular systems biology learn from one another? Genome Res. *25*, 1466–1472.

Mohamed, T.M.A., Stone, N.R., Berry, E.C., Radzinsky, E., Huang, Y., Pratt, K., Ang, Y.-S., Yu, P., Wang, H., Tang, S., et al. (2017). Chemical Enhancement of In Vitro and In Vivo Direct Cardiac Reprogramming. Circulation *135*, 978–995.

Morris, S.A., Cahan, P., Li, H., Zhao, A.M., Roman, A.K.S., Shivdasani, R.A., Collins, J.J., and Daley, G.Q. (2014). Dissecting Engineered Cell Types and Enhancing Cell Fate Conversion via CellNet. Cell *158*, 889–902.

Nam, Y.-J., Song, K., Luo, X., Daniel, E., Lambeth, K., West, K., Hill, J.A., DiMaio, J.M., Baker, L.A., Bassel-Duby, R., et al. (2013). Reprogramming of human fibroblasts toward a cardiac fate. Proc. Natl. Acad. Sci. U. S. A. *110*, 5588–5593.

Nam, Y.-J., Lubczyk, C., Bhakta, M., Zang, T., Fernandez-Perez, A., McAnally, J., Bassel-Duby, R., Olson, E.N., and Munshi, N.V. (2014). Induction of diverse cardiac cell types by reprogramming fibroblasts with cardiac transcription factors. Development *141*, 4267–4278.

Niepel, M., Hafner, M., Mills, C.E., Subramanian, K., Williams, E.H., Chung, M., Gaudio, B., Barrette, A.M., Stern, A.D., Hu, B., et al. (2019). A Multi-center Study on the Reproducibility of Drug-Response Assays in Mammalian Cell Lines. Cell Syst *9*, 35–48.e5.

Olson, E.N., and Srivastava, D. (1996). Molecular pathways controlling heart development. Science *272*, 671–676.

Padovan-Merhar, O., Nair, G.P., Biaesch, A.G., Mayer, A., Scarfone, S., Foley, S.W., Wu, A.R., Churchman, L.S., Singh, A., and Raj, A. (2015). Single Mammalian Cells Compensate for Differences in Cellular Volume and DNA Copy Number through Independent Global

Transcriptional Mechanisms. Mol. Cell 1–36.

Palpant, N.J., Pabon, L., Roberts, M., Hadland, B., Jones, D., Jones, C., Moon, R.T., Ruzzo, W.L., Bernstein, I., Zheng, Y., et al. (2015). Inhibition of β-catenin signaling respecifies anterior-like endothelium into beating human cardiomyocytes. Development *142*, 3198–3209.

Parekh, U., Wu, Y., Zhao, D., Worlikar, A., Shah, N., Zhang, K., and Mali, P. (2018). Mapping Cellular Reprogramming via Pooled Overexpression Screens with Paired Fitness and Single-Cell RNA-Sequencing Readout. Cels *0*.

Potthoff, M.J., Arnold, M.A., McAnally, J., Richardson, J.A., Bassel-Duby, R., and Olson, E.N. (2007). Regulation of skeletal muscle sarcomere integrity and postnatal muscle function by Mef2c. Mol. Cell. Biol. *27*, 8143–8151.

Qian, L., Berry, E.C., Fu, J.-D., Ieda, M., and Srivastava, D. (2013). Reprogramming of mouse fibroblasts into cardiomyocyte-like cells in vitro. Nat. Protoc. *8*, 1204–1215.

Rackham, O.J.L., Firas, J., Fang, H., Oates, M.E., Holmes, M.L., Knaupp, A.S., FANTOM Consortium, Suzuki, H., Nefzger, C.M., Daub, C.O., et al. (2016). A predictive computational framework for direct reprogramming between human cell types. Nat. Genet.

Raj, A., van den Bogaard, P., Rifkin, S.A., van Oudenaarden, A., and Tyagi, S. (2008). Imaging individual mRNA molecules using multiple singly labeled probes. Nat. Methods *5*, 877–879.

Shaffer, S.M., Dunagin, M.C., Torborg, S.R., Torre, E.A., Emert, B., Krepler, C., Beqiri, M., Sproesser, K., Brafford, P.A., Xiao, M., et al. (2017). Rare cell variability and drug-induced reprogramming as a mode of cancer drug resistance. Nature.

Shanmughapriya, S., Tomar, D., Dong, Z., Slovik, K.J., Nemani, N., Natarajaseenivasan, K., Carvalho, E., Lu, C., Corrigan, K., Garikipati, V.N.S., et al. (2018). FOXD1-dependent MICU1 expression regulates mitochondrial activity and cell differentiation. Nat. Commun. *9*, 3449.

Shi, G., and Jin, Y. (2010). Role of Oct4 in maintaining and regaining stem cell pluripotency. Stem Cell Res. Ther. *1*, 39.

Shishkin, A.A., Giannoukos, G., Kucukural, A., Ciulla, D., Busby, M., Surka, C., Chen, J., Bhattacharyya, R.P., Rudy, R.F., Patel, M.M., et al. (2015). Simultaneous generation of many RNA-seq libraries in a single reaction. Nat. Methods *12*, 323–325.

Szalai, B., Subramanian, V., Holland, C.H., Alföldi, R., Puskás, L.G., and Saez-Rodriguez, J. (2019). Signatures of cell death and proliferation in perturbation transcriptomics data-from confounding factor to effective prediction. Nucleic Acids Res. *47*, 10010–10026.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663–676.

Takeuchi, J.K., and Bruneau, B.G. (2009). Directed transdifferentiation of mouse mesoderm to heart tissue by defined factors. Nature *459*, 708–711.

Targoff, K.L., Colombo, S., George, V., Schell, T., Kim, S.-H., Solnica-Krezel, L., and Yelon, D. (2013). Nkx genes are essential for maintenance of ventricular identity. Development *140*,

4203–4213.

Tomaru, Y., Hasegawa, R., Suzuki, T., Sato, T., Kubosaki, A., Suzuki, M., Kawaji, H., Forrest, A.R.R., Hayashizaki, Y., FANTOM Consortium, et al. (2014). A transient disruption of fibroblastic transcriptional regulatory network facilitates trans-differentiation. Nucleic Acids Res. *42*, 8905–8913.

Vaquerizas, J.M., Kummerfeld, S.K., Teichmann, S.A., and Luscombe, N.M. (2009). A census of human transcription factors: function, expression and evolution. Nat. Rev. Genet. *10*, 252–263.

Vierbuchen, T., Ostermeier, A., Pang, Z.P., Kokubu, Y., Südhof, T.C., and Wernig, M. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. Nature *463*, 1035–1041.

Wallace, E.W.J., and Beggs, J.D. (2017). Extremely fast and incredibly close: cotranscriptional splicing in budding yeast. RNA *23*, 601–610.

Wang, L., Liu, Z., Yin, C., Asfour, H., Chen, O., Li, Y., Bursac, N., Liu, J., and Qian, L. (2015). Stoichiometry of Gata4, Mef2c, and Tbx5 influences the efficiency and quality of induced cardiac myocyte reprogramming. Circ. Res. *116*, 237–244.

Weintraub, H., Tapscott, S.J., Davis, R.L., Thayer, M.J., Adam, M.A., Lassar, A.B., and Miller, A.D. (1989). Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc. Natl. Acad. Sci. U. S. A. *86*, 5434–5438.

Yu, G., Wang, L.-G., Han, Y., and He, Q.-Y. (2012). clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS *16*, 284–287.

Zhou, H., Morales, M.G., Hashimoto, H., Dickson, M.E., Song, K., Ye, W., Kim, M.S., Niederstrasser, H., Wang, Z., Chen, B., et al. (2017). ZNF281 enhances cardiac reprogramming by modulating cardiac and inflammatory gene expression. Genes Dev. *31*, 1770–1783.

Zhu, W.-Z., Xie, Y., Moyes, K.W., Gold, J.D., Askari, B., and Laflamme, M.A. (2010). Neuregulin/ErbB Signaling Regulates Cardiac Subtype Specification in Differentiating Human Embryonic Stem CellsNovelty and Significance. Circ. Res. *107*, 776–786.

Zhu, W.-Z., Van Biber, B., and Laflamme, M.A. (2011). Methods for the derivation and use of cardiomyocytes from human pluripotent stem cells. Methods Mol. Biol. *767*, 419–431.

Figure Legends

Figure 1: Perturbation panel profiling of iPSC-derived cardiac myocytes. A) Conceptual guide for different perturbation-response models of lineage-maintaining genes. In the “pillars” model, lineage-maintaining genes are relatively unresponsive to perturbation, whereas non-lineage-related genes do respond. In the “springs” model, lineage-maintaining genes are

more often responsive to perturbation than other genes. B) Perturbation panel profiling experiments and analysis: parallel cultures of human iPSC-derived cardiac myocytes (iPSC-CM), exposed to 75 different perturbation conditions in triplicate (see Table S1) or vehicle control. C) Parallel RNA extraction and library preparation for RNAtag-seq of perturbed and control samples followed by differential expression analysis. Per gene, differential expression in each condition relative to vehicle control samples followed by quantification of the number of conditions in which differential expression was observed. Representative results for two genes, *FOXJ3* and *MEF2C*, are shown. D) Responsiveness to perturbation analysis of all expressed transcription factor genes. All transcription factor genes expressed at 20 RPM or greater in controls were considered (grey, histogram). Pre-registered set of cardiac myocyte lineage-establishing and -maintaining genes shown in red. For each gene, the number of conditions in which any differential expression (top) differential expression down (middle) or up (bottom). E) Static expression feature analysis of all expressed transcription factor genes. All transcription factor genes expressed at 20 RPM or greater in controls were considered (grey, histogram). iPSC-derived cardiac myocyte-specificity score is Jensen-Shannon specificity to iPSC-derived cardiac myocytes without GTEx Skin and Heart (See Methods). Pre-registered set of cardiac myocyte lineage-establishing and maintaining genes shown in red. For each gene, the average expression level in vehicle control samples (top) or iPSC-derived cardiac myocyte-specificity score (bottom, see Methods). F) Comparison of responsiveness (number of iPSC-CM conditions in which up-regulated) and mean expression (mean RPM in control iPSC-CMs) for all expressed transcription factor genes. Vertical-only jitter added for visualization of all points with the same responsiveness value. G) Comparison of responsiveness and iPSC-derived cardiac myocyte-specificity scores for all expressed transcription factor genes. Vertical-only jitter added for visualization of all points with the same responsiveness value. Quadrant-separating lines ("arbitrary cutoffs") chosen as elbows of the cumulative distribution function of each feature over all transcription factor genes expressed at 20 RPM or greater in controls. H) Schematic of cardiac transdifferentiation experimental workflow. See Methods for full details. I) Representative images of *GAPDH*, *NPPA*, and *TNNT2* smFISH of 7F-transduced fibroblasts after 24 days, demonstrating a cell that expresses marker genes *NPPA* and *TNNT2* at high levels and one cell that does not. Cells called "marker-positive" if *TNNT2* spots > 19 or *NPPA* spots > 24, thresholds chosen arbitrarily. 0 cells in negative control MXs-empty condition are "marker-positive". Scale bars are 10 μm.

Figure 2: Suppression of fibroblast-responsive genes in a directed change of cell identity. A) P^3 schematic (top) and responsiveness results (bottom) for primary human GM00942 fibroblasts (primary fibroblasts) exposed to 75 different perturbation conditions and vehicle control. All transcription factors expressed at 20 RPM or greater in vehicle control fibroblasts (grey histogram). Selected responsive genes used in later reprogramming experiments shown in green. B) Comparison of responsiveness and primary fibroblast-specificity score per gene (Jensen-Shannon specificity to primary fibroblasts without GTEx Heart and Skin, See Methods). Quadrant-separating lines chosen as elbows of the cdf of each feature over all transcription factor genes expressed at 20 RPM or greater in controls. Transcription factor barriers to reprogramming are 8 of 16 factors tested found to have a

significant effect (Fisher's exact test p-value < 0.05) on colony count against controls. See Methods for a full list of factors tested. C) Schematic of experimental workflow for hiF-T fibroblast reprogramming experiments. In parallel, hiF-Ts were transduced with shRNAs targeting genes perturbable in fibroblasts (3 shRNAs per target, each cultured in technical triplicate per experimental replicate), expanded in culture for 6 days, counted, and seeded in parallel on MEF feeders. Dox-mediated OKSM induction for 20 days prior to fixation and analysis of reprogrammed colonies. D) Representative fluorescence microscopy images of Alkaline Phosphatase activity (Vector Red staining, 4X magnification, 555 nm wavelength channel) in reprogrammed hiF-Ts under different knockdown conditions after 20 days of OKSM induction. Activity shown in black, as indicated by red arrowheads. Scale bar 5 mm. E) Colony counts with alkaline phosphatase activity after reprogramming for representative experimental replicates for two batches of perturbable target genes. F) Further characterization of hiF-T-iPSC colonies after reprogramming under different knockdown conditions. 10X magnification brightfield images (top 2 rows), Alkaline Phosphatase activity by Vector Red (middle 2 rows), and Tra-1-60 expression as identified by immunofluorescence (bottom 2 rows) shown for the same wells and inset colonies. Scale bar 5 mm for wells, 200 μm for colonies.

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Supplementary Figure 2: Exploratory analysis of RNAtag-seq results of perturbed iPSC-derived cardiac myocytes, primary fibroblasts, and HeLa cells. A) Heatmap of $\log_2$(TPM+0.01) expression values for all transcription factor genes expressed at 5 TPM or greater on average across all quality-controlled samples. B) Principal Component Analysis of all genes expressed at 5 TPM or greater on average across all quality-controlled samples.

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Supplementary Figure 6: Gene set enrichment analysis of perturbed iPSC-derived cardiac myocyte and primary fibroblast samples. Gene set over-enrichment analysis was performed for all Gene Ontology - Cellular Component terms. For each cell type (A,B: iPSC-derived cardiac myocyte; C,D: primary fibroblast) genes were filtered first to all genes expressed at 20 RPM or greater in controls. GO terms were tested for over-enrichment in the set of filtered genes that are frequently up-regulated (up in 4 or more conditions, A,C) relative to all filtered genes, as well

as in the set of filtered genes that are infrequently up- or down-regulated (up or down in 1 or 0 conditions total, B,D) relative to all filtered genes.

Supplementary Figure 7: Transcription factor gene expression specificity in iPSC-derived cardiac myocytes. Expression specificity scores for heart (A,B) and iPSC-derived cardiac myocytes (C,D) are plotted on the x axis. Analyses were repeated on datasets including (A,C) or excluding (B,D) the most similar samples. Responsiveness to perturbation, i.e., the number of conditions in which a gene was expressed higher than in controls, is on the y axis. Panel D also appears in Figure 1.

Supplementary Figure 8: P^3 analysis of perturbed primary fibroblasts. A) Responsiveness to perturbation (i.e., number of conditions in which up-regulated in GM00942 primary fibroblasts) against average expression in control GM00942 primary fibroblasts. B) Responsiveness to perturbation against fibroblast-specificity of expression (JS_{sp} score in GM00942 primary fibroblasts, excluding GTEx Skin and Heart samples, See Methods).

Supplementary Figure 9: Transcription factor gene expression specificity in fibroblast samples. Expression specificity scores for Skin (A,B) and control GM00942 primary fibroblasts (C,D) are plotted on the x axis (JSsp specificity scores, see Methods). Analyses were repeated on datasets including (A,C) or excluding (B,D) the most similar samples. Responsiveness to perturbation, i.e., the number of conditions in which a gene was expressed higher than in controls, is on the y axis. Panel D also appears in Figure 2.

Supplementary Figure 10: iPSC reprogramming efficiency of hiF-T cells after knockdown of fibroblast-responsive factors. A) Schematic representation of experimental workflow. B, C) Representative replicates. Control shRNAs (1 = empty backbone, 2 = scrambled negative control, 3 = Luciferase-targeting negative control). Controls shown in same row as factors tested are matched per batch. Alkaline Phosphatase-positive colony counts per well counted in triplicate per shRNA. Blue lines correspond to average colony count per well across all control wells in that replicate. Three shRNAs per factor tested. Representative RT-qPCR based assessment of knockdown efficiency.

Supplementary Figure 11: Additional replicates of iPSC reprogramming of hiF-T cells after knockdown of fibroblast-responsive transcription factors. Control shRNAs (1 = empty backbone, 2 = scrambled negative control, 3 = Luciferase-targeting negative control). Controls shown in same graph as factors tested are matched per batch. Alkaline Phosphatase-positive colony counts per well counted in triplicate per shRNA. Three shRNAs per factor tested. Second experimental replicates (A,B), and third experimental replicate (C) for all barriers to fibroblast reprogramming shown in Fig. 2. Blue lines correspond to average colony count per well across all control wells in that replicate. Each experimental replicate was conducted using different vials of cells and different batches of media and feeder mouse embryonic fibroblasts (MEFs) on different days; knockdown samples are compared against controls in the same batch. Reprogramming rate variability was highest in the third replicate for unknown reasons.

Supplementary Figure 12: Evaluating cardiac transdifferentiation after overexpression of iPSC-derived cardiac myocyte-responsive transcription factors in fibroblasts. A) Selection of 7 transcription factors found to be frequently up-regulated after perturbation in iPSC-derived cardiac myocytes (iPSC-CM; see Methods). B) Schematic of experimental workflow: iCard-responsive factors (the 7UP cocktail: *SP3, ZBTB10, ZBTB44, NFIA, SSH2, ZNF770, ZFP91*) were cloned into pMXs retroviral expression vectors, the same system as 7F. 10^4 immHCF fibroblasts per sample were seeded, transduced with empty pMXs vehicle control (MXs-empty), 7F factors only, 7F + 7UP factors, or 7UP factors only, and cultured in parallel for 24 days (See Methods). C) On day 24, an smFISH scan (1 plane of focus per field of view) was conducted to estimate expression of cardiac myocyte marker genes TNNT2 and NPPA for 671 - 1156 cells per condition. Per cell, we counted the number of TNNT2 and NPPA transcripts per cell, and per-gene quadrant thresholds were chosen arbitrarily to be at least 2 transcripts greater than the maximum expression in MXs-empty condition.

Discussion: Our model predicted that responsive factors would be important for lineage maintenance. We also wanted to test whether responsive factors could in fact help establish lineage. Thus, we asked whether the addition of responsive factors to an already established transdifferentiation cocktail would further enhance transdifferentiation. We found that the overexpression of 7UP factors did not enhance 7F-mediated cardiac transdifferentiation, nor was 7UP overexpression alone sufficient for cardiac transdifferentiation. The conclusion would be that the addition of factors to the source cell type that were identified by P^3 in the target cell type do not help enhance directed changes in cell type. This conclusion fits with our overarching hypothesis, which is that factors identified by P3 are important for lineage maintenance (rather than specification). We do note, however, that there are a number of technical limitations that may potentially explain the lack of enhancement. First, each factor is overexpressed by a single virus, so the quantity of virus required to achieve high MOI for all factors could itself be toxic (there is twice as much virus per cell in 7F+7UP as in 7F alone). Additionally, it is possible that the timing of overexpression of 7UP factors relative to the addition of 7F could be important for reprogramming, because as putative lineage-maintainers, 7UP factors may increase transdifferentiation efficiency only after a cell has begun the process of identity specification but may interfere with that specification if expressed before 7F has begun to act on a cell. Also, it may be that the stoichiometry of the factors is critical for the enhancement of reprogramming, which is difficult to control experimentally, especially when a large number of factors are added simultaneously.

Figure 1

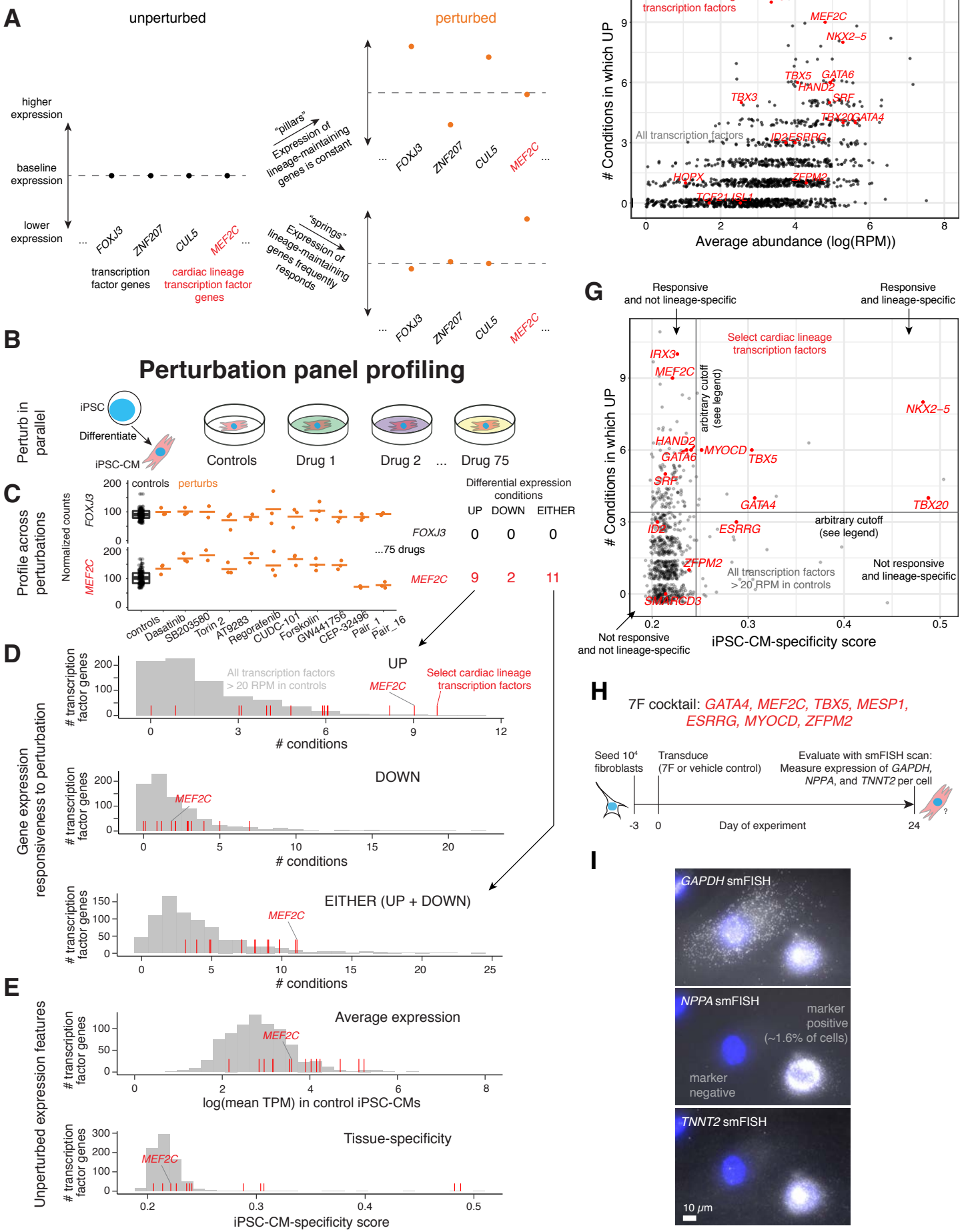
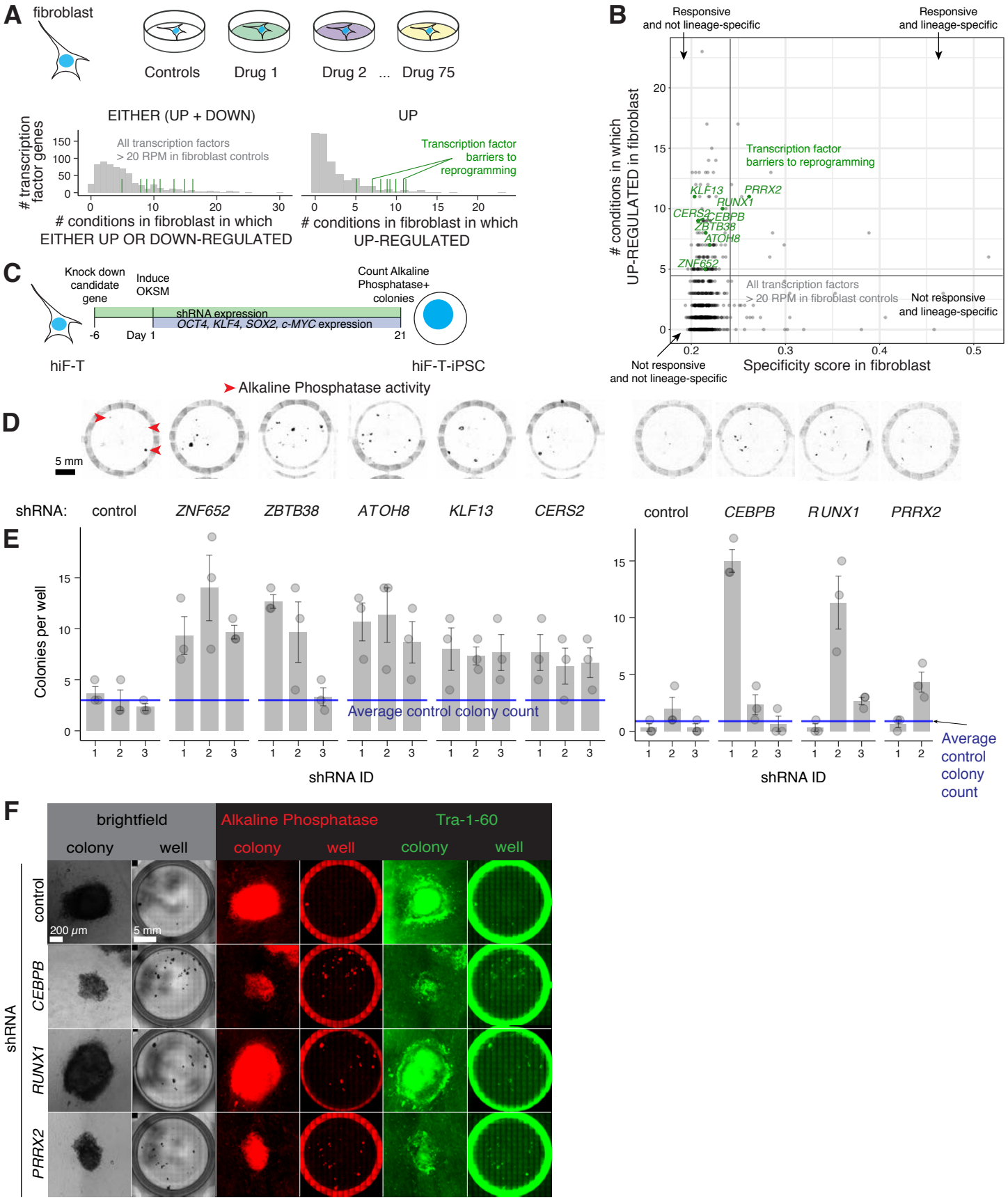
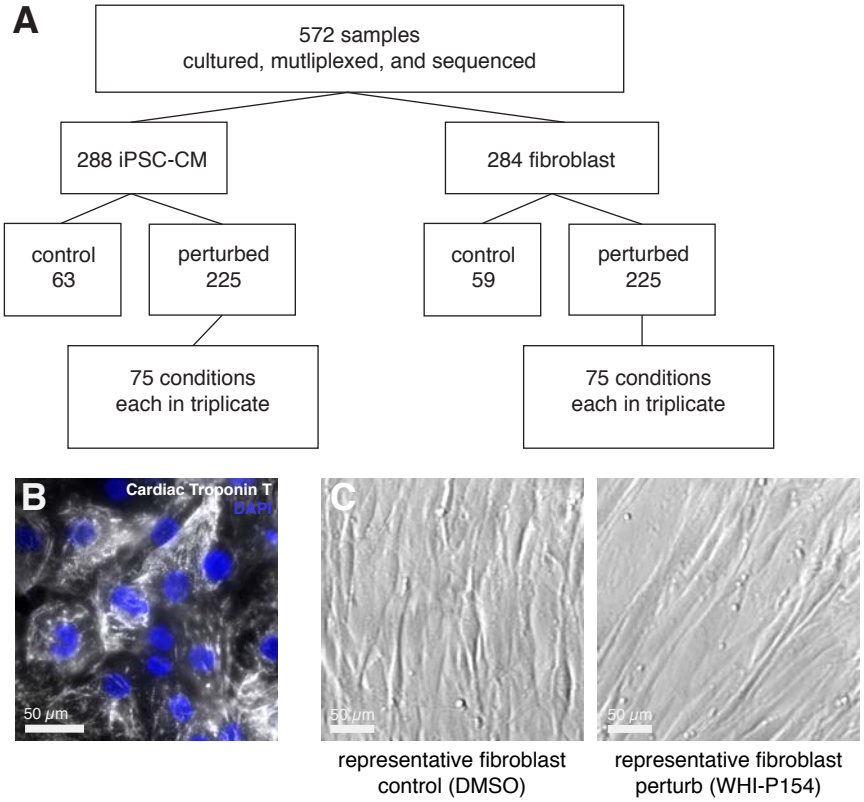

A
unperturbed
perturbed
higher expression
baseline expression
lower expression
FOXJ3
ZNF207
CUL5
MEF2C
transcription factor genes
cardiac lineage transcription factor genes
"pillars" Expression of lineage-maintaining genes is constant
"springs" Expression of lineage-maintaining genes frequently responds
B
Perturbation panel profiling
Perturb in parallel
iPSC
Differentiate
iPSC-CM
Controls
Drug 1
Drug 2
Drug 75
C
Profile across perturbations
controls
perturbs
Normalized counts
...75 drugs
Differential expression conditions
UP DOWN EITHER
FOXJ3 0 0 0
MEF2C 9 2 11
Dasatinib
SB203580
Torin 2
AT9283
Regoratenib
CUDC-101
Forskolin
GW441756
CEP-32496
Pair_1
Pair_16
D
Gene expression responsiveness to perturbation
UP
All transcription factors > 20 RPM in controls
Select cardiac lineage transcription factors
transcription factor genes
conditions
DOWN
EITHER (UP + DOWN)
E
Unperturbed expression features
Average expression
log(mean TPM) in control iPSC-CMs
Tissue-specificity
iPSC-CM-specificity score
F
Select cardiac lineage transcription factors
All transcription factors
Conditions in which UP
Average abundance (log(RPM))
IRX3
MEF2C
NKX2–5
TBX5
GATA6
HAND2
TBX3
SRF
TBX20
GATA4
ID2
ESRRG
HOPX
ZFPM2
TCF21
ISL1
G
Responsive and not lineage-specific
Responsive and lineage-specific
Select cardiac lineage transcription factors
arbitrary cutoff (see legend)
MYOCD
ID2
ZFPM2
SMARCD3
All transcription factors > 20 RPM in controls
Not responsive and lineage-specific
Not responsive and not lineage-specific
iPSC-CM-specificity score
H
7F cocktail: GATA4, MEF2C, TBX5, MESP1, ESRRG, MYOCD, ZFPM2
Seed 10⁴ fibroblasts
Transduce (7F or vehicle control)
Evaluate with smFISH scan: Measure expression of GAPDH, NPPA, and TNNT2 per cell
Day of experiment
I
GAPDH smFISH
NPPA smFISH
marker positive (~1.6% of cells)
marker negative
TNNT2 smFISH
10 μm

Figure 2


A
fibroblast
Controls
Drug 1
Drug 2 ... Drug 75
EITHER (UP + DOWN)
UP
transcription factor genes
All transcription factors
> 20 RPM in fibroblast controls
Transcription factor barriers to reprogramming
conditions in fibroblast in which EITHER UP OR DOWN-REGULATED
conditions in fibroblast in which UP-REGULATED
B
Responsive and not lineage-specific
Responsive and lineage-specific
conditions in which UP-REGULATED in fibroblast
Transcription factor barriers to reprogramming
KLF13
PRRX2
RUNX1
CERS2
CEBPB
ZBTB38
ATOH8
ZNF652
All transcription factors
> 20 RPM in fibroblast controls
Not responsive and lineage-specific
Not responsive and not lineage-specific
Specificity score in fibroblast
C
Knock down candidate gene
Induce OKSM
Count Alkaline Phosphatase+ colonies
shRNA expression
OCT4, KLF4, SOX2, c-MYC expression
-6
Day 1
21
hiF-T
hiF-T-iPSC
Alkaline Phosphatase activity
D
5 mm
shRNA: control ZNF652 ZBTB38 ATOH8 KLF13 CERS2 control CEBPB RUNX1 PRRX2
E
Colonies per well
Average control colony count
shRNA ID
Average control colony count
F
brightfield
Alkaline Phosphatase
Tra-1-60
colony
well
shRNA
control
CEBPB
RUNX1
PRRX2
200 μm
5 mm

Supplementary Figure 1



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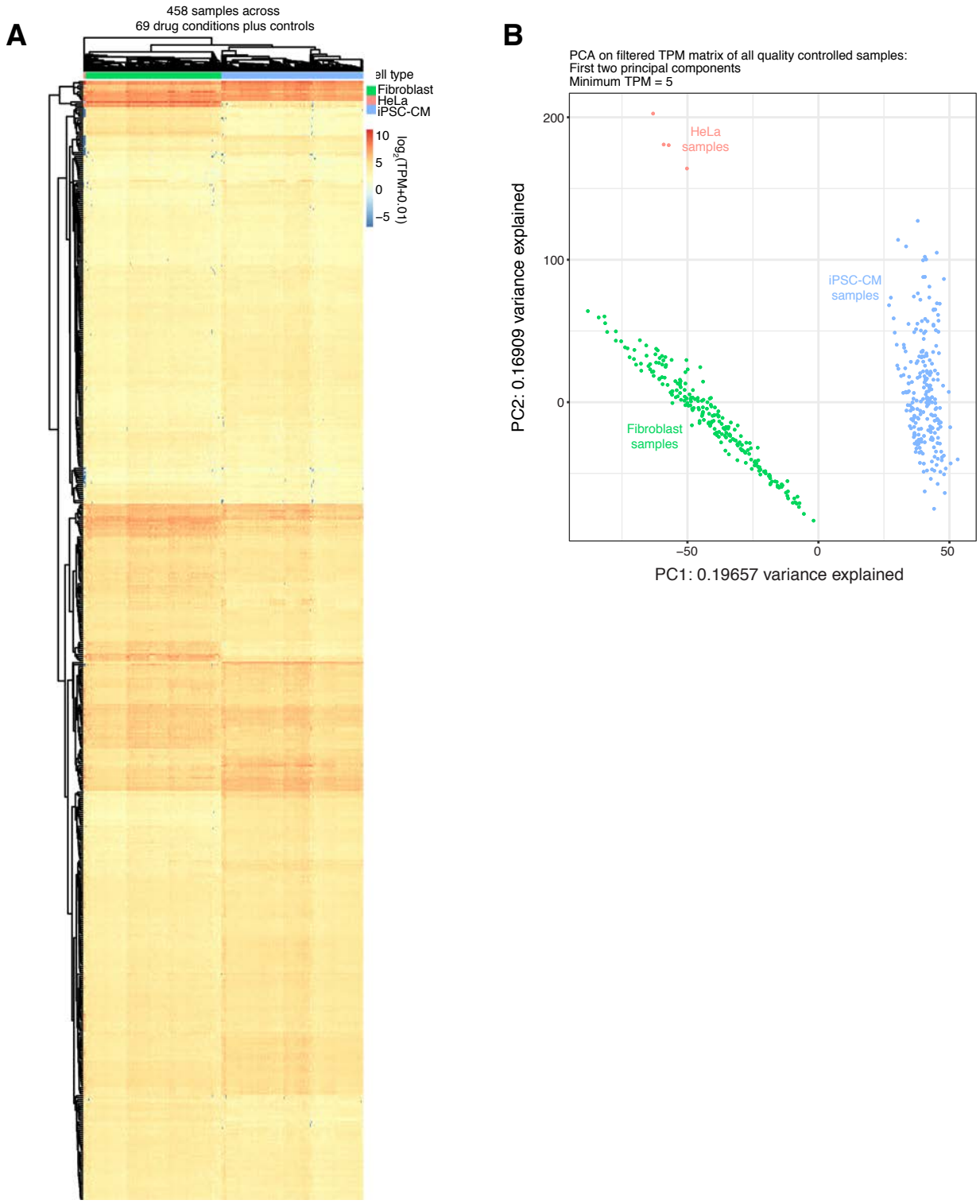

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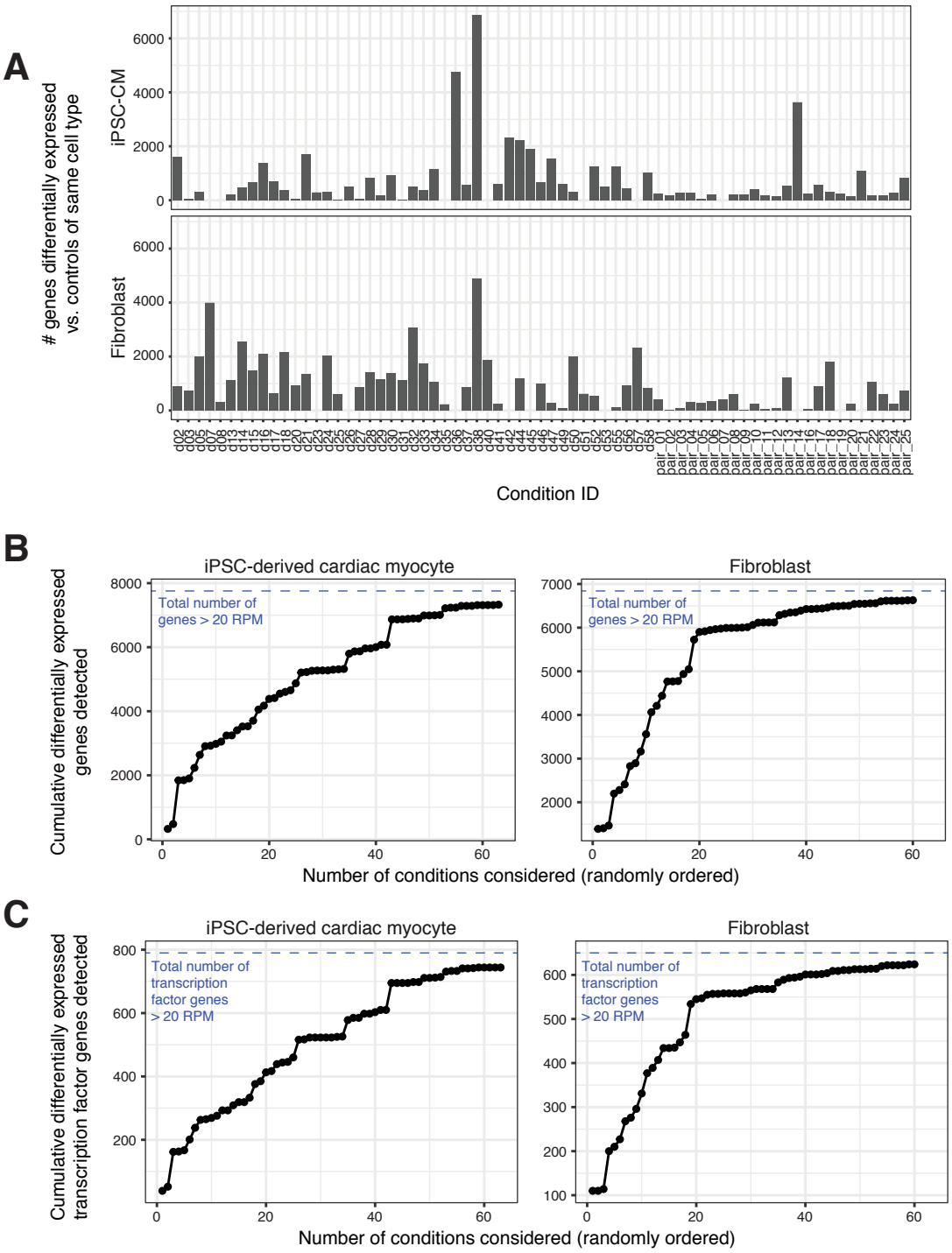

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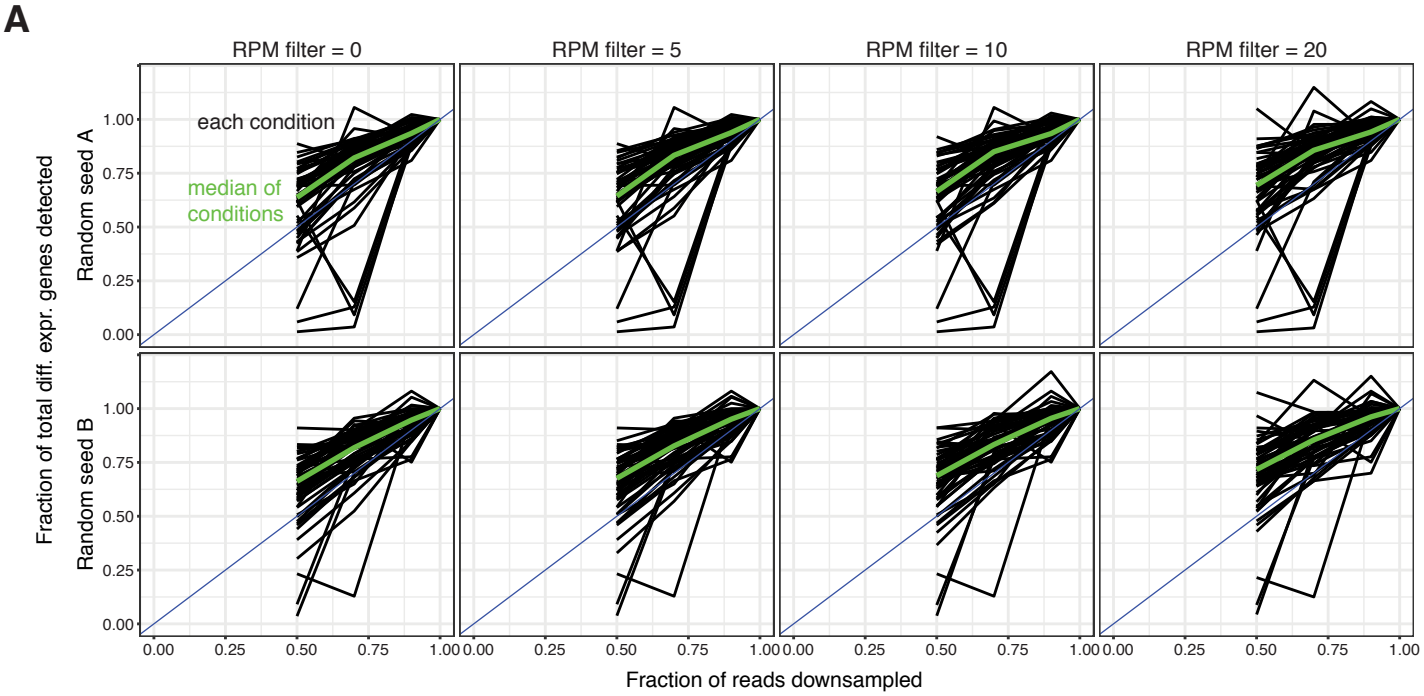

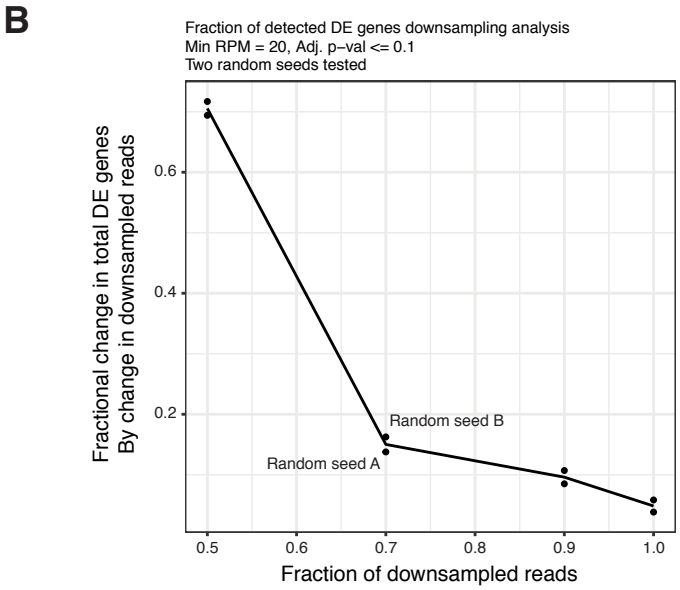

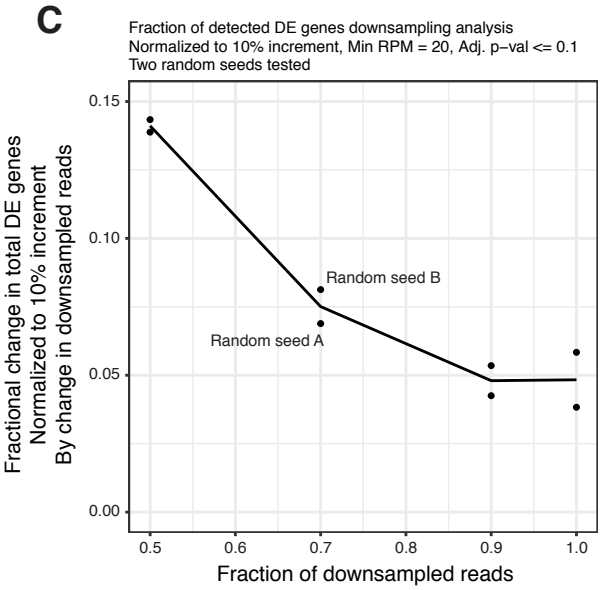

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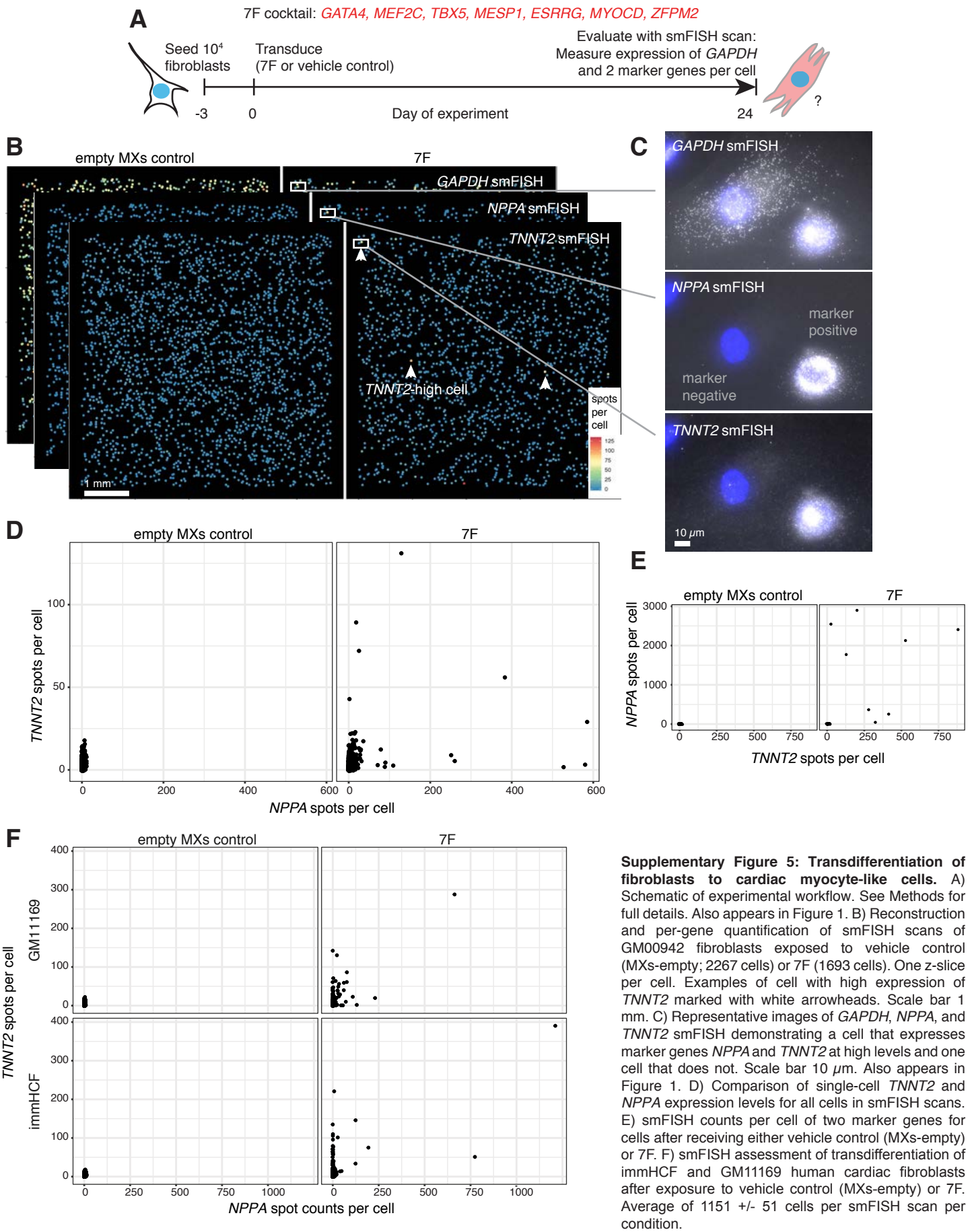

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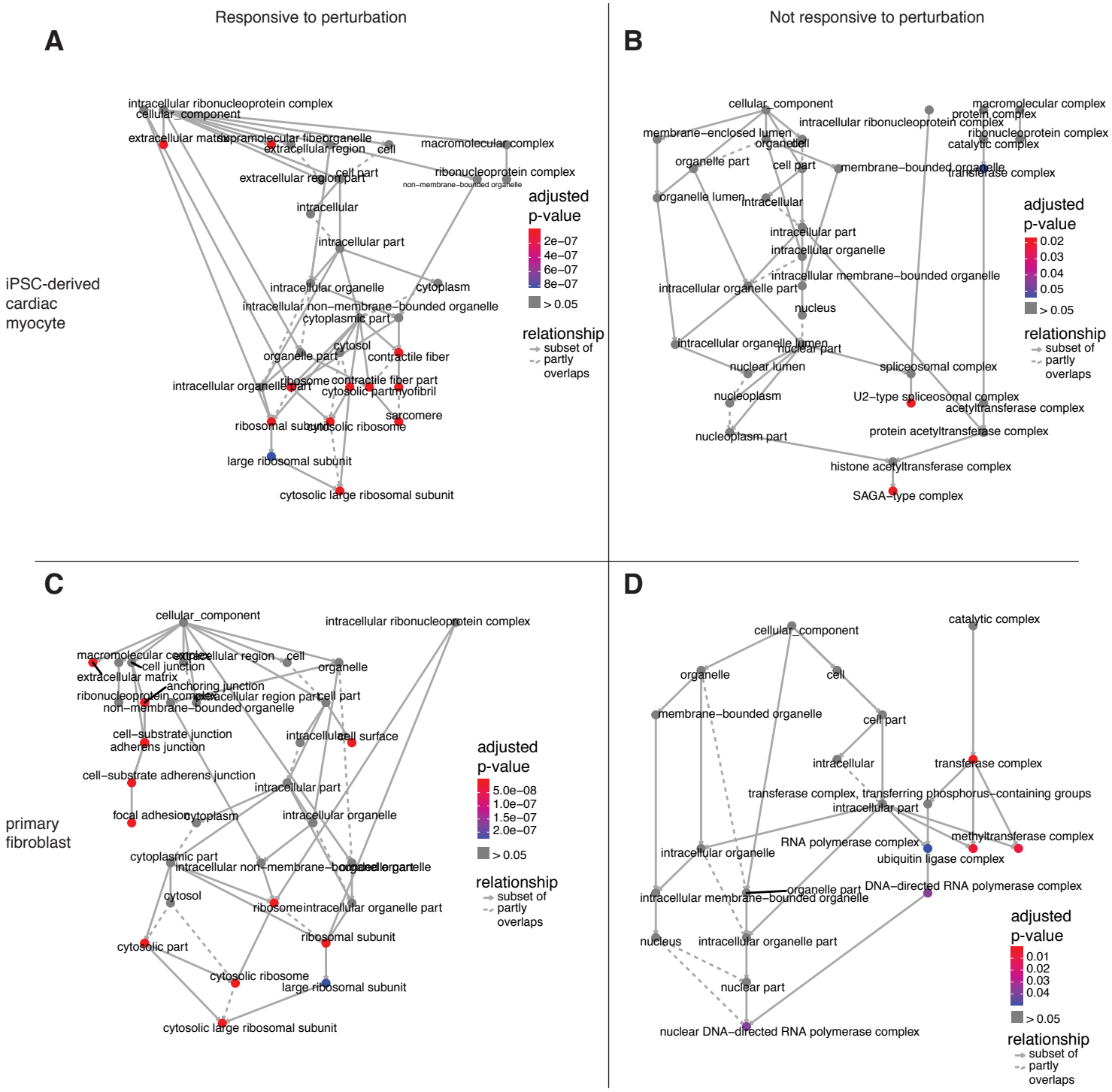

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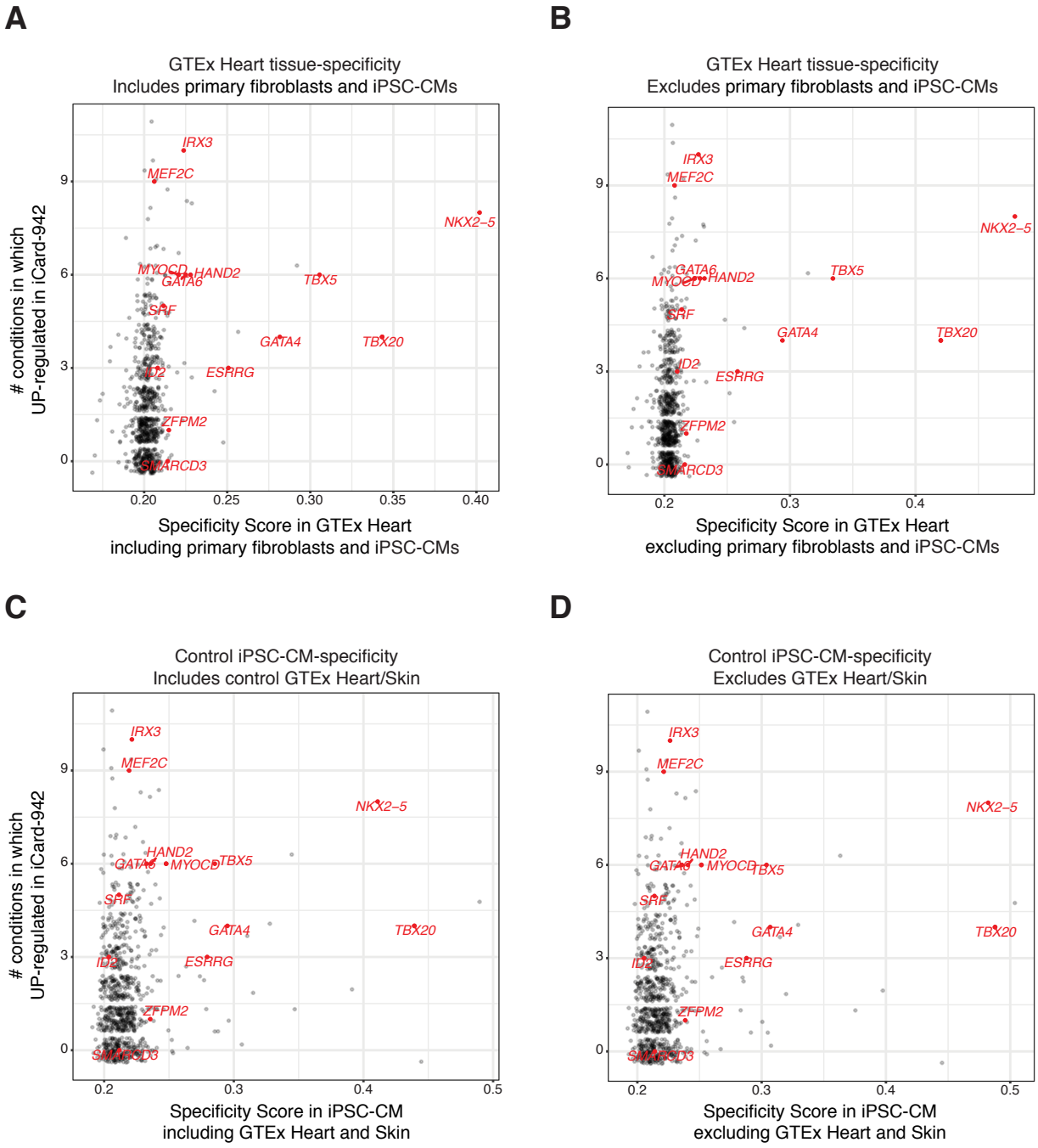

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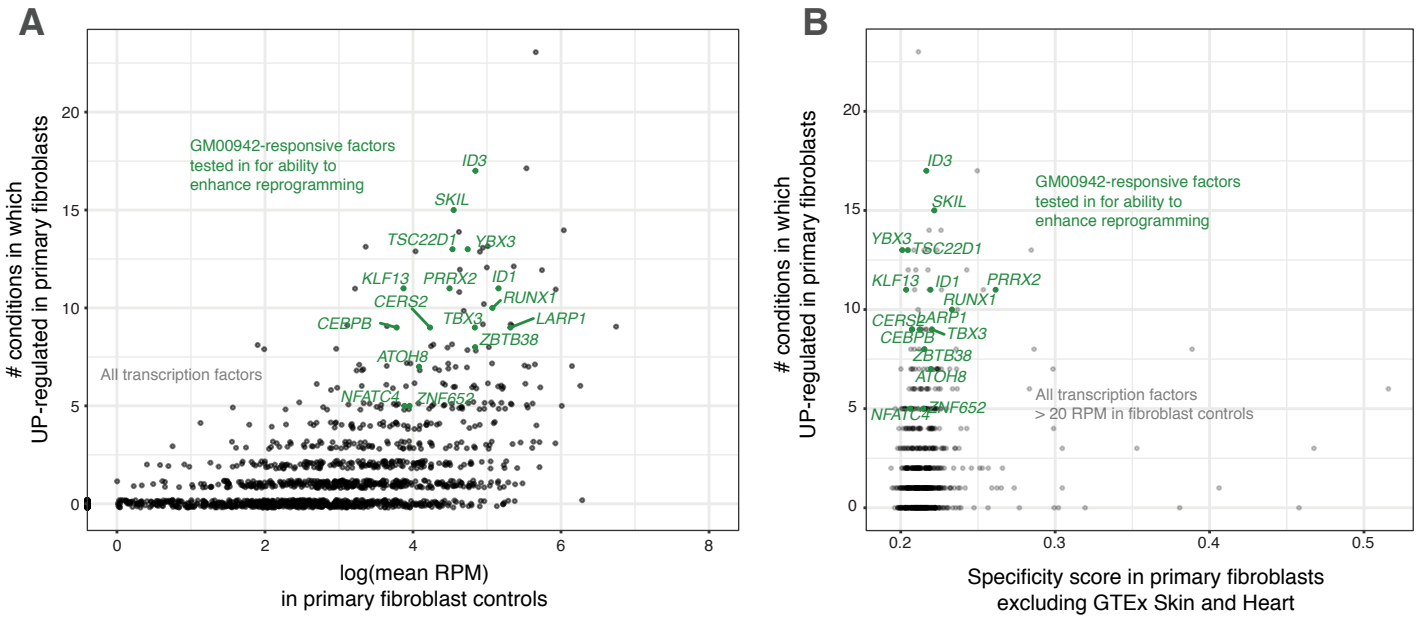
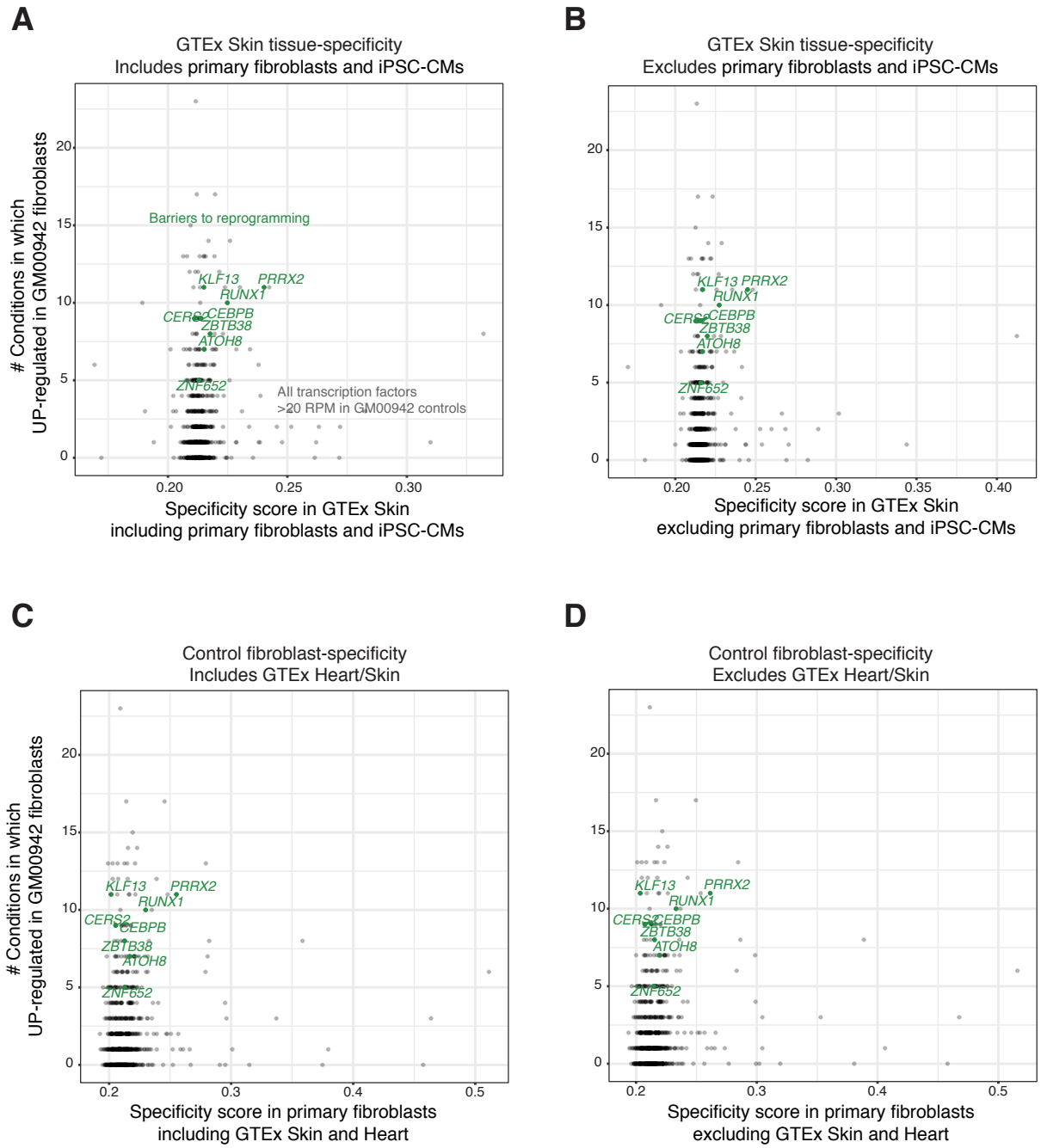
Supplementary Figure 8



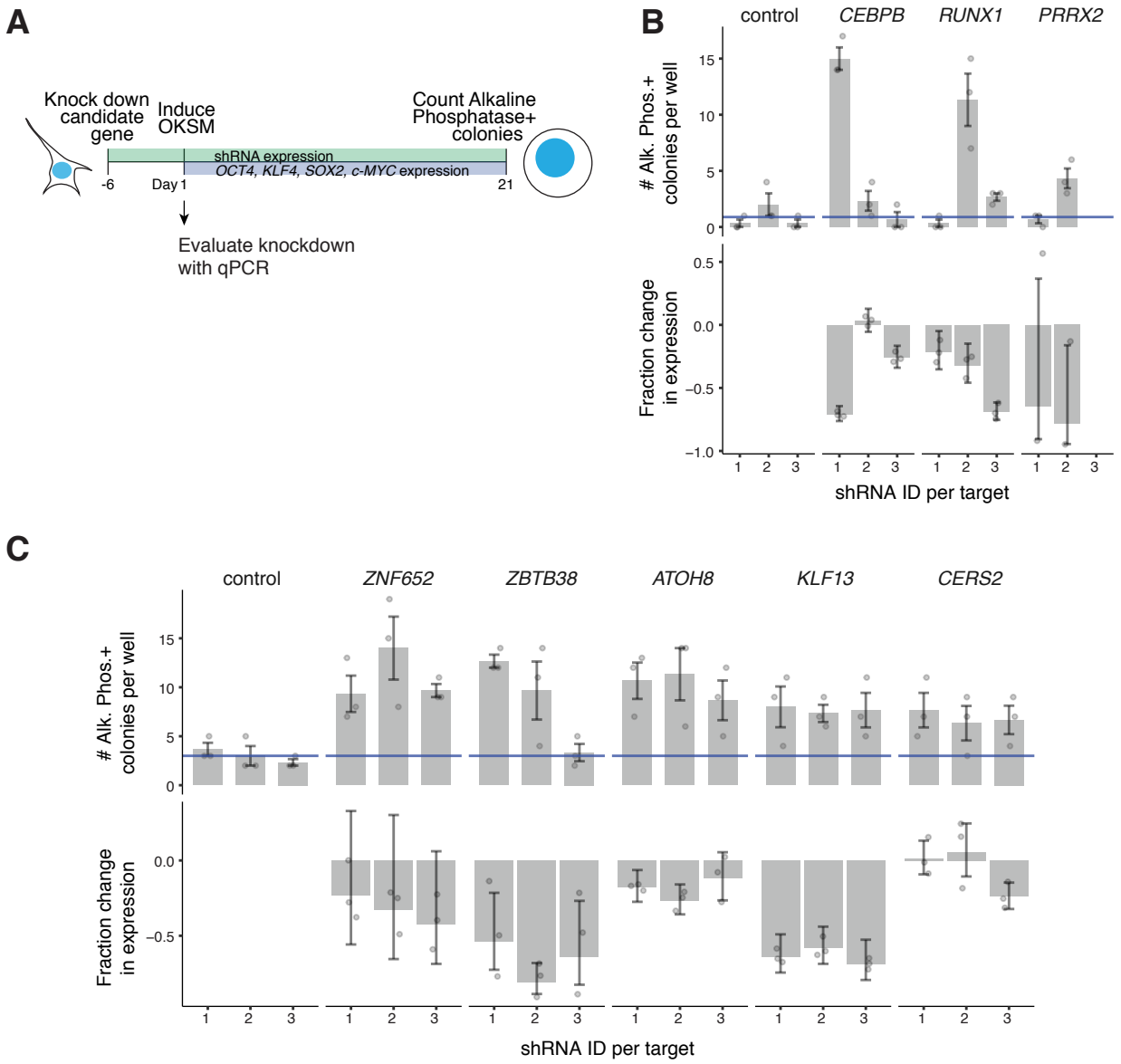
Supplementary Figure 8: P³ analysis of perturbed primary fibroblasts. A) Responsiveness to perturbation (i.e., number of conditions in which up-regulated in GM00942 primary fibroblasts) against average expression in control GM00942 primary fibroblasts. B) Responsiveness to perturbation against fibroblast-specificity of expression (JS_{sp} score in GM00942 primary fibroblasts, excluding GTEx Skin and Heart samples, See Methods).

Supplementary Figure 9



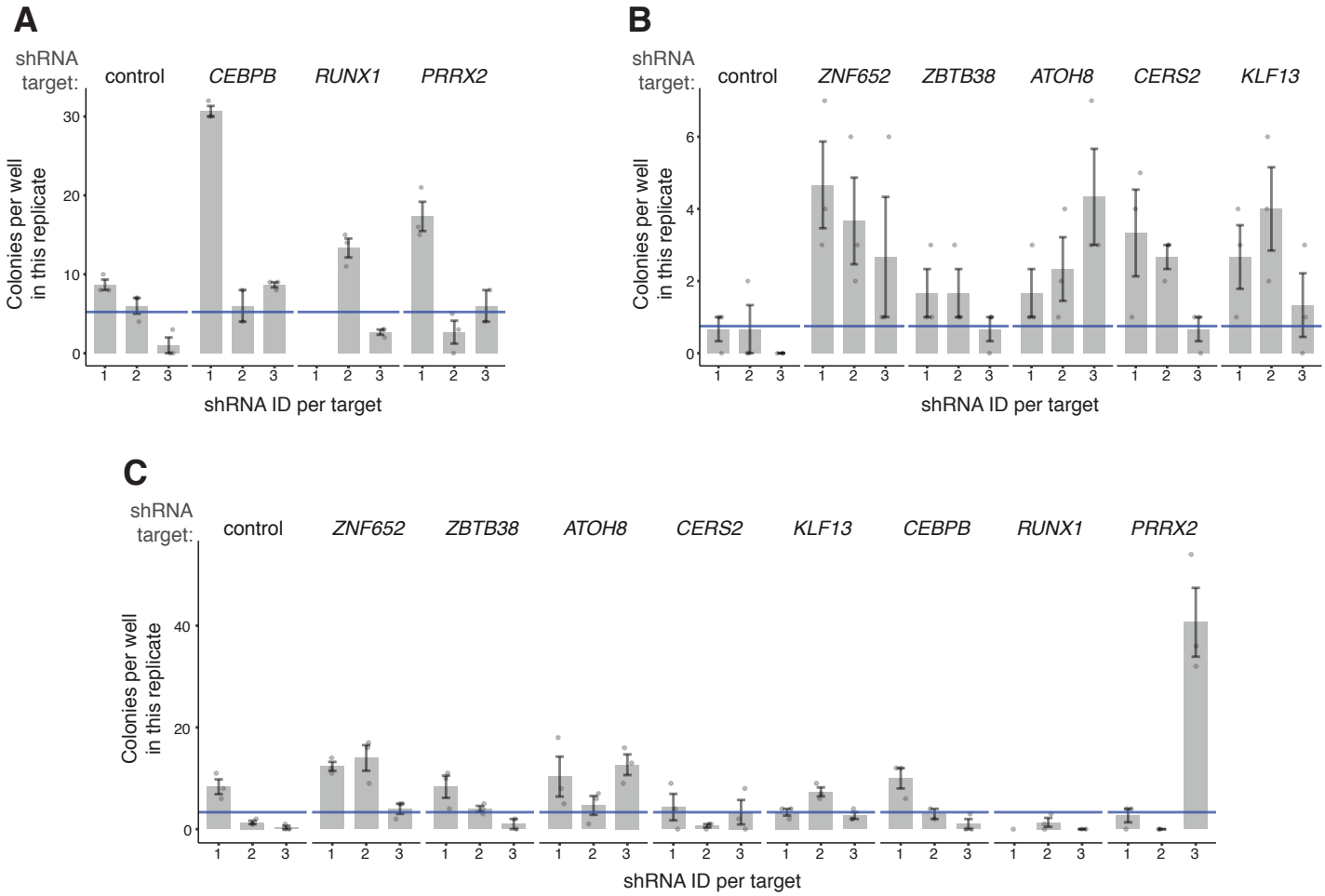
Supplementary Figure 9: Transcription factor gene expression specificity in fibroblast samples. Expression specificity scores for Skin (A,B) and control GM00942 primary fibroblasts (C,D) are plotted on the x axis (JS_{sp} specificity scores, see Methods). Analyses were repeated on datasets including (A,C) or excluding (B,D) the most similar samples. Responsiveness to perturbation, i.e., the number of conditions in which a gene was expressed higher than in controls, is on the y axis. Panel D also appears in Figure 2.

Supplementary Figure 10



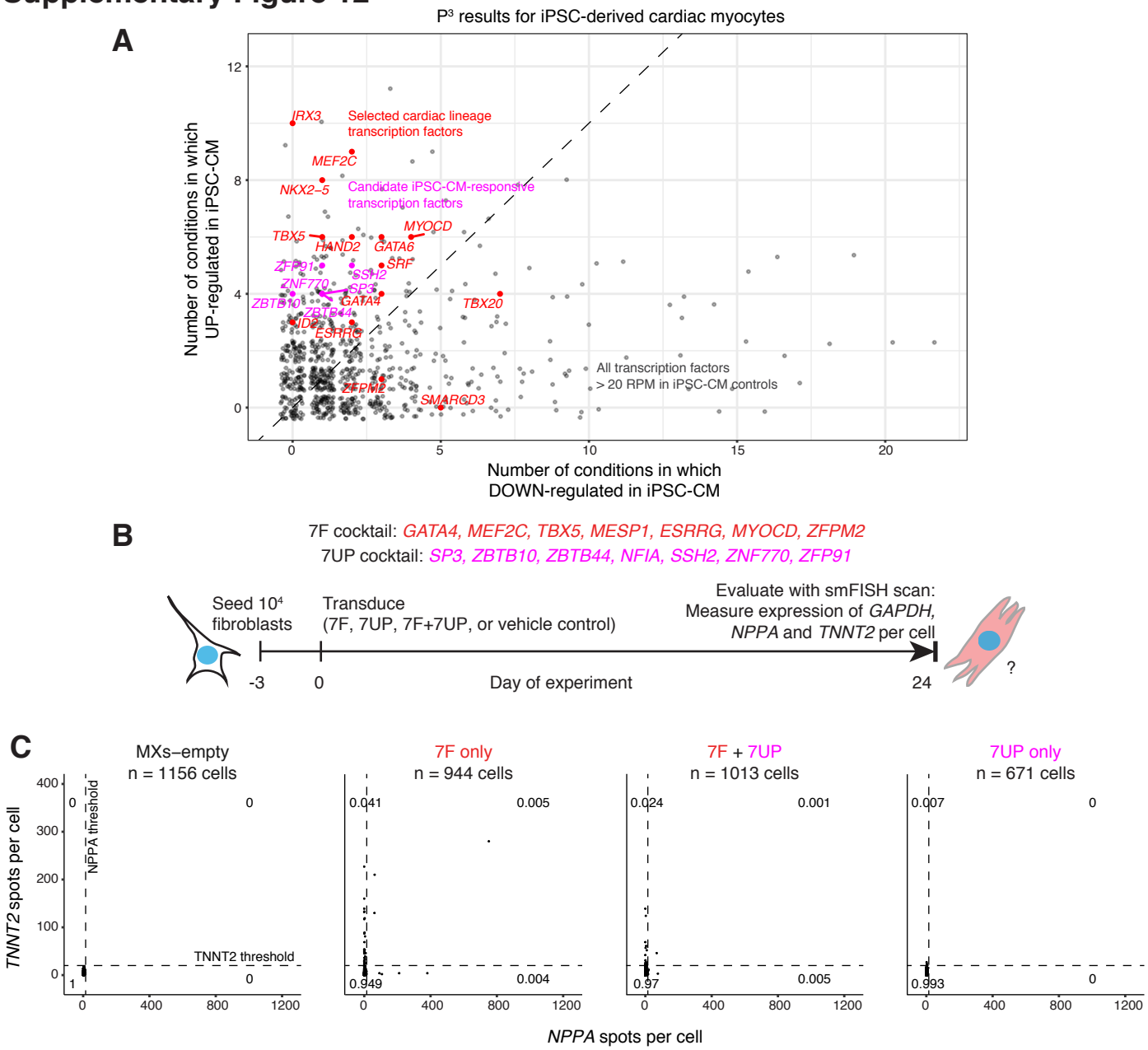
Supplementary Figure 10: iPSC reprogramming efficiency of hiF-T cells after knockdown of fibroblast-responsive factors. A) Schematic representation of experimental workflow. B, C) Representative replicates. Control shRNAs (1 = empty backbone, 2 = scrambled negative control, 3 = Luciferase-targeting negative control). Controls shown in same row as factors tested are matched per batch. Alkaline Phosphatase-positive colony counts per well counted in triplicate per shRNA. Blue lines correspond to average colony count per well across all control wells in that replicate. Three shRNAs per factor tested. Representative RT-qPCR based assessment of knockdown efficiency.

Supplementary Figure 11



Supplementary Figure 11: Additional replicates of iPSC reprogramming of hiF-T cells after knockdown of fibroblast-responsive transcription factors. Control shRNAs (1 = empty backbone, 2 = scrambled negative control, 3 = Luciferase-targeting negative control). Controls shown in same graph as factors tested are matched per batch. Alkaline Phosphatase-positive colony counts per well counted in triplicate per shRNA. Three shRNAs per factor tested. Second experimental replicates (A,B), and third experimental replicate (C) for all barriers to fibroblast reprogramming shown in Fig. 2. Blue lines correspond to average colony count per well across all control wells in that replicate. Each experimental replicate was conducted using different vials of cells and different batches of media and feeder mouse embryonic fibroblasts (MEFs) on different days; knockdown samples are compared against controls in the same batch. Reprogramming rate variability was highest in the third replicate for unknown reasons.

Supplementary Figure 12



Supplementary Figure 12: Evaluating cardiac transdifferentiation after overexpression of iPSC-derived cardiac myocyte-responsive transcription factors in fibroblasts. A) Selection of 7 transcription factors found to be frequently up-regulated after perturbation in iPSC-derived cardiac myocytes (iPSC-CM; see Methods). B) Schematic of experimental workflow: iCard-responsive factors (the 7UP cocktail: *SP3, ZBTB10, ZBTB44, NFIA, SSH2, ZNF770, ZFP91*) were cloned into pMXs retroviral expression vectors, the same system as 7F. 10^4 immHCF fibroblasts per sample were seeded, transduced with empty pMXs vehicle control (MXs-empty), 7F factors only, 7F + 7UP factors, or 7UP factors only, and cultured in parallel for 24 days (See Methods). C) On day 24, an smFISH scan (1 plane of focus per field of view) was conducted to estimate expression of cardiac myocyte marker genes *TNNT2* and *NPPA* for 671 - 1156 cells per condition. Per cell, we counted the number of *TNNT2* and *NPPA* transcripts per cell, and per-gene quadrant thresholds were chosen arbitrarily to be at least 2 transcripts greater than the maximum expression in MXs-empty condition.

Discussion: Our model predicted that responsive factors would be important for lineage maintenance. We also wanted to test whether responsive factors could in fact help establish lineage. Thus, we asked whether the addition of responsive factors to an already established transdifferentiation cocktail would further enhance transdifferentiation. We found that the overexpression of 7UP factors did not enhance 7F-mediated cardiac transdifferentiation, nor was 7UP overexpression alone sufficient for cardiac transdifferentiation. The conclusion would be that the addition of factors to the source cell type that were identified by P3 in the target cell type do not help enhance directed changes in cell type. This conclusion fits with our overarching hypothesis, which is that factors identified by P3 are important for lineage maintenance (rather than specification). We do note, however, that there are a number of technical limitations that may potentially explain the lack of enhancement. First, each factor is overexpressed by a single virus, so the quantity of virus required to achieve high MOI for all factors could itself be toxic (there is twice as much virus per cell in 7F+7UP as in 7F alone). Additionally, it is possible that the timing of overexpression of 7UP factors relative to the addition of 7F could be important for reprogramming, because as putative lineage-maintainers, 7UP factors may increase transdifferentiation efficiency only after a cell has begun the process of identity specification but may interfere with that specification if expressed before 7F has begun to act on a cell. Also, it may be that the stoichiometry of the factors is critical for the enhancement of reprogramming, which is difficult to control experimentally, especially when a large number of factors are added simultaneously.