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Review

Tools and Concepts for Interrogating and Defining Cellular Identity

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Defining the mechanisms that generate specialized cell types and coordinate their functions is critical for understanding organ development and renewal. New tools and discoveries are challenging and refining our definitions of a cell type. A rapidly growing toolkit for single-cell analyses has expanded the number of markers that can be assigned to a cell simultaneously, revealing heterogeneity within cell types that were previously regarded as homogeneous populations. Additionally, cell types defined by specific molecular markers can exhibit distinct, context-dependent functions; for example, between tissues in homeostasis and those responding to damage. Here we review the current technologies used to identify and characterize cells, and we discuss how experimental and pathological perturbations are adding increasing complexity to our definitions of cell identity.

Understanding how specialized cells work together to ensure tissue and organ function is a central objective of developmental and stem cell biology, and a critical step toward achieving this goal is to comprehensively catalog the cells that make up a tissue. Cells can be categorized according to their features, such as molecular markers, or according to their function (Figure 1). Feature- and function-based definitions are tightly linked; identifying features associated with a cell type is essential to assess its function(s) and to define the signaling pathways, regulatory logic, and cellular structures that endow those functions. In addition, shared cellular features can provide hints of shared functions in systems for which direct functional assessments are challenging, including humans. In recent years, our capacity to define cellular features has exploded, with rapid advances in single-cell profiling generating a wealth of high-resolution, high-dimensional data that establish transcriptional, epigenetic, and proteomic signatures of cells. These analyses are revealing previously unrecognized heterogeneity and are reshaping our understanding of cellular identity.

Current studies are seeking to generate integrated definitions for cell types that encompass features and functions, but an enduring challenge is that the contribution of a cell to a tissue varies according to its context. Thus, a specific feature, such as a molecular marker, may correlate with a particular function in one context, but feature and function can become uncoupled when the context is altered. For example, because of a phenomenon called cell competition, cells with mutations that appear neutral in one context can be selectively eliminated when combined with wild-type neighbors, or wild-type cells can be outcompeted by "super-fit" cells. Even in contexts in which cells are genetically identical, heterogeneity in the surrounding signals can privilege certain cells so that cells in close proximity to morphogens or niche factors expand at the expense of more distal cells.

Moreover, cell types that are stable during homeostasis in the adult can perform new functions and/or acquire new features under damage or disease conditions, a phenomenon called plasticity. For example, cells that have made fate commitments can revert to less differentiated states (de-differentiation) or directly convert to a mature cell type of a distinct lineage (trans-differentiation). In many cases, cell type transitions arise during perturbations that alter a cell's interaction with its microenvironment. Environmental and pathological perturbations can alter cellular microenvironments, as can many experimental strategies for cell type analysis that remove a cell from its native context and expose it to a new microenvironment, including through transplantation or *ex vivo* culture models. Thus, a key goal for establishing a comprehensive understanding of cell identity is to distinguish what a cell type does in steady state from what it is capable of doing in a given environment.

As a result of these recent studies of cellular features and functions, the term "cell type" has acquired multiple meanings and interpretations (Clevers et al., 2017). Cell type categories that depend on hard-wired functions or on functions that invariably track with specific features are being called into question. We are still in the early stages of comprehensively categorizing the cells in a tissue in homeostasis according to any one molecular layer (transcriptome, cell surface markers, chromatin architecture, and so on), and ongoing efforts are seeking to connect these molecular maps of tissues to the underlying mechanisms of tissue function. Particular challenges include defining the functional consequences of unearthed heterogeneities and determining how the categories these approaches identify correspond to cellular transitions along differentiation trajectories in

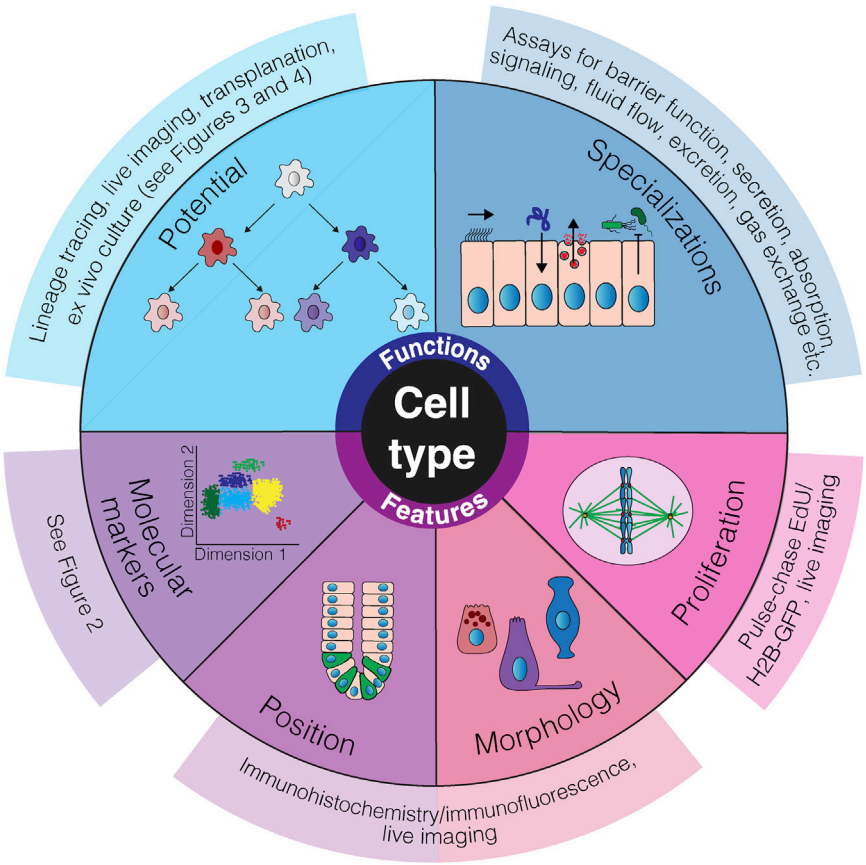

Figure 1. Defining Cell Types
Shown are attributes used to categorize cells into types (inner wedges) and strategies to assay them (outer wedges). Cells can be classified according to their functions (top), and their physical features (bottom).

real time. We are also facing a need to generate experimental and computational frameworks to integrate cellular profiles generated with different modalities, and we are challenged to reconcile discrepancies between the groupings of cells they define. New contexts and stimuli—such as injuries, diseases, aging, and environmental factors—will further refine these pictures or perhaps upend them.

This review aims to present the diversity of frameworks from which to approach the problem of cell categorization, the tools available to pursue them, and concepts and challenges to consider in their interpretation and synthesis, with a predominant focus on mammalian epithelial tissues. We first synthesize and assess the strategies to categorize cells based on their features and functions. We then delve deeper into cellular function and how cells exhibiting a specific feature or set of features can exhibit different functions in different contexts. We discuss key findings regarding the assessment of stem cell function and cell plasticity, in which cells exhibit expanded or altered functional repertoires following experimental manipulation or damage. Finally, we consider how cellular context can drive selective elimination or expansion of certain cells through cell competition. This work highlights the complex interplay between intrinsic and extrinsic properties that endow and coordinate cellular functions.

Tools for Assessing Cellular Features and Functions

In this section, we review the wide and rapidly expanding toolkit that is increasing the scale and precision with which tissues can be deconstructed into their component cell types. We focus on advances in pursuit of three major goals: (1) Detecting features associated with a cell type from a pre-defined list of candidates, (2) identifying new features and cell types through unbiased approaches, and (3) defining cellular relationships.

Goal 1: Detection of Features Associated with a Cell Type from a Pre-defined List of Candidates

Distinguishing cells based on a limited number of pre-selected features, such as morphology or expression of a set of specific genes or proteins, is a long-standing and powerful approach to distinguish and isolate cell types. Approaches to detect candidate features continue to play critical roles in understanding cell identity even as unbiased -omics profiling approaches expand, in part because of trade-offs such as cost and ease of implementation. Moreover, defining a limited suite of identifying markers plays a critical role in further downstream characterization of a cell type; for example, through genetic perturbations. Ongoing efforts are developing tools capable of detecting an increasing number of candidates in a single sample (Figure 2A).

Microscopy has been a powerful tool for discrimination between cell types for over a century. Early work distinguished cells based on their morphology and dye-staining properties (Ehrlich, 1877; Golgi, 1885). In the mid-1900s, technologies emerged that allowed cells to be detected based on molecular features: proteins could be detected with antibodies by immunohistochemistry (Coons et al., 1941) and nucleic acids with complementary sequence probes by *in situ* hybridization (ISH; Gall and Pardue, 1969)). These probes can be conjugated to enzymes that produce a colored precipitate for detection by bright-field microscopy or to fluorophores, which allow them to be detected *in situ* using a fluorescence microscope, or in dissociated cells by flow cytometry. Fluorescent proteins further expand this toolkit by allowing genetic labeling and live imaging of proteins (Rodriguez et al., 2017) and protein-RNA complexes (Bertrand et al., 1998; Nelles et al., 2016) (Figures 2Ai and 2Aiv).

Compared with colorimetric approaches, fluorescence approaches increase the number of features that can be detected in a cell simultaneously by labeling each detection reagent with distinct fluorophores. However, although the number of available dyes and fluorescent proteins for labeling is large, spectral overlap between fluorophores frequently limits the number of features that can be distinguished. Efforts are ongoing to increase the number

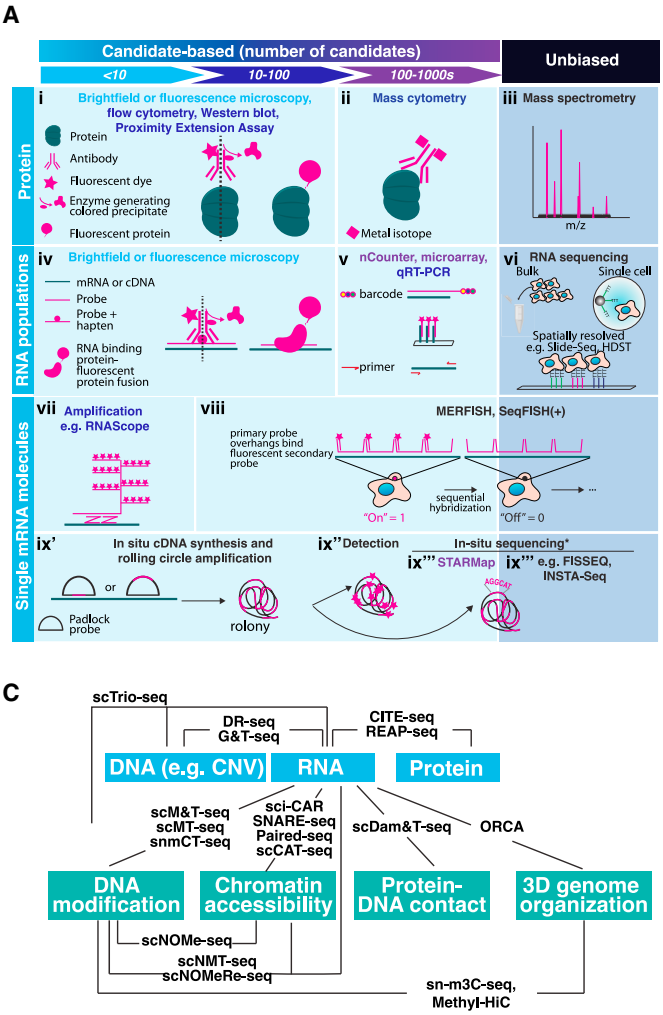

Figure 2. Strategies to Detect Molecular Features Associated with a Cell Type

(A) Common approaches to detect proteins (i–iii) or RNAs (iv–ix) associated with cells. Strategies are grouped according to whether they require upfront selection of candidates (left columns) and/or allow unbiased profiling of the proteome or transcriptome (right column). The approximate range of features that can be detected by each strategy is indicated by color, corresponding to the blue-to-purple scale. Targets are indicated in teal; detection reagents are indicated in pink. (i–iii) Approaches for protein detection. (iv) Microscopic approaches for RNA detection. (v) Approaches to quantify a panel of mRNAs from populations of cells. (vi) Unbiased approaches to detect RNAs. (vii–viii) Approaches to detect single mRNA molecules by ISH. (viii) The primary probe that directly binds the sequence can be fluorescently labeled, or the sequence can be first bound with an unlabeled primary probe containing overhangs, which function as landing pads for hybridization of fluorescent secondary "readout" probes. For MERFISH, the pattern of on/off fluorescence observed for a given RNA over multiple rounds of hybridization reveals its binary barcode (readout probe bound = 1; not bound = 0). (ix) Approaches to detect single mRNA molecules *in situ* by first generating rolling circle-amplified cDNA. *, *in situ* sequencing can be used for candidate-based approaches to read out barcode sequences or for unbiased approaches to read out short sequences of the transcripts.

(B) Common approaches to profile epigenomic features associated with a cell type. **, strategies currently available to profile at the single-cell level.

(C) Approaches to simultaneously profile more than one modality in a sample.

of proteins that can be assessed in a single sample by microscopy or flow cytometry, particularly using spectral approaches with linear unmixing, which distinguish fluorophores according to their signature emission patterns across the spectrum instead of isolating specific wavelength ranges (Valm et al., 2017; Zimmermann, 2005). Alternatively, repeated cycles of antibody staining, signal removal, and re-staining with new antibodies can further increase the number of proteins that can be detected (Gerdes et al., 2013; Lin et al., 2015; Pirici et al., 2009). An expanded suite of proteins can also be detected by conjugating antibodies to DNA barcodes, which are then iteratively revealed by addition of corresponding fluorescent (oligo)nucleotides (Goltsev et al., 2018; Saka et al., 2019) or by photocleavage of the oligo spot by spot and subsequent analysis (commercialized as Digital Spatial Profiling [DSP]; Merritt et al., 2019).

Highly multiplexed protein profiling can also be achieved by conjugating antibodies to non-biological metal isotopes (commonly lanthanides) instead of fluorophores, which are then detected by mass spectrometry (Figure 2Aii). This allows detection of, in principle, 100 or more targets simultaneously (Bandura et al., 2009; Bendall et al., 2011). This approach, called mass cytometry (commercialized as CyTOF), can detect features on dissociated cells in a manner analogous to fluorescence flow cytometry. For example, in recent work, the expression of 73 proteins was evaluated in 26 million tumor and non-tumor cells to profile human breast cancer (Wagner et al., 2019). This approach has since been extended beyond cell surface antigens, allowing immune cells to be distinguished based on their global histone modification profiles (EpiTOF; Cheung et al., 2018) as well as to assess features of cellular metabolism (single-cell metabolic profiling [scMEP]; Hartmann et al., 2020). Mass cytometry not only allows profiling of dissociated cells but can also be applied to detect proteins in intact tissue sections in a manner analogous to immunofluorescence. In imaging mass cytometry (Giesen et al., 2014; commercialized as Hyperion), fixed tissue is labeled with lanthanide-conjugated antibodies and ablated with a high-resolution laser spot by spot. Each ablated spot is then transferred to the CyTOF for analysis, allowing detection of more than 30 epitopes with spatial resolution. Recent work has extended this approach to allow simultaneous detection of proteins and mRNA (Schulz et al., 2018). In multiplexed ion beam imaging (MIBI; Angelo et al., 2014; commercialized as MIBIscope), a focused ion beam is used to scan across the sample, liberating secondary ions from the lanthanides for detection by the mass spectrometer. The relative advantages of these approaches are reviewed elsewhere (Bodenmiller, 2016).

Whereas multiplexed protein detection strategies can report on tens to hundreds of features per sample, multiplexed RNA detection approaches tens of thousands. A subset of these approaches, such as microarrays and the NanoString nCounter (Geiss et al., 2008), multiplex quantification of mRNA levels from homogenized populations of cells, increasing the number of targets that can be detected compared with quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) (Figure 2Av). Alternatively, individual mRNAs can be detected directly in fixed cells, providing single-cell resolution. These approaches generate sufficient signal for detection of single RNA molecules by tiling numerous labeled probes along the target sequence (Femino et al., 1998; Raj et al., 2008; Figure 2Aviii), or utilizing a small number of probes and amplifying the signal through either scaffolding (Player et al., 2001; Wang et al., 2012; Kishi et al., 2019; Figure 2Avii) or rolling circle amplification (Larsson et al., 2010; Figure 2Aix).

To multiplex these approaches, each mRNA sequence is assigned an identifying barcode that can be read out by *in situ* sequencing (Figure 2Aix) or fluorescence ISH (FISH) (Figure 2Aviii). For *in situ* sequencing approaches, the barcode is a short nucleotide sequence that is introduced into the cDNA amplicon during rolling circle amplification and read out by sequencing by ligation (Figure 2Aix). The earliest iteration of this approach allowed detection of 39 transcripts in tissue sections (Ke et al., 2013). The recently developed STARmap technique built on this approach using a modified amplification process and sequencing-by-ligation approach as well as crosslinking of the amplicons within a tissue hydrogel. Together, these developments increased the detection efficiency over earlier *in situ* sequencing approaches, allowing STARmap to detect transcripts from over 1,000 genes and in tissue sections up to 150 μm thick (Wang et al., 2018). As an alternative to introducing the barcode during rolling circle amplification, cells can first be barcoded with a virus, and the barcode RNA can be read out by *in situ* sequencing (BAR-seq), an approach used recently to identify the projection patterns of individual neurons (Chen et al., 2019).

A number of techniques have also been developed for detecting individual RNA molecules by FISH (Codeluppi et al., 2018; Levsky et al., 2002; Lubeck and Cai, 2012). For sequential FISH (seqFISH; Lubeck et al., 2014) and multiplexed error-robust FISH (MERFISH; Chen et al., 2015), each mRNA is assigned an ordered sequence of fluorophores or on/off fluorescence as a barcode (Figure 2Aviii). This fluorophore is read out over sequential rounds of hybridization, imaging, and disruption of the hybrid or photobleaching. However, a challenge exists for these sequential imaging approaches. During each round of imaging, occasionally a spot that should fluoresce fails to be detected, or, conversely, stray probes or autofluorescence cause a spot that should not fluoresce during that imaging round to be misidentified as positive. As a result, the sequence that is ultimately read out may be incorrect, potentially in such a way that one barcode is misidentified as another. To address this, MERFISH incorporates an RNA encoding scheme based on Hamming distance, the number of errors that would convert one barcode into another (Chen et al., 2015). For example, a barcode library with a Hamming distance of 2 requires that 2 errors occur for one barcode to be mis-identified as another. These approaches were initially applied in cultured cells and subsequently extended to tissues (Moffitt et al., 2016; Shah et al., 2016). A major limitation for increasing the detection of mRNAs to the transcriptome level is the high density of RNAs within a cell, leading to overlap between detected spots. SeqFISH and MERFISH have recently addressed this challenge by hybridizing only a subset of the RNAs in the cell with a given color at a given time (SeqFISH+; Eng et al., 2019) or physically expanding the specimen with expansion microscopy (MERFISH; Xia et al., 2019). These approaches allow detection of probes targeting 10,000 genes, a dramatic step toward unbiased profiling of gene expression *in situ*.

Broadly, these diverse techniques for analysis of pre-selected candidate features are subject to a variety of tradeoffs. For example, preserving spatial context can reveal new cellular relationships shaping cell identity, but it may come at the cost of throughput compared with techniques using dissociated cells. In addition, in many cases, as the number of features that can be detected increases (Figure 2A), so, too, does the challenge of executing the experiment in terms of reagent costs, equipment, and requirements for technical expertise. For example, lower feature numbers are often detectable with equipment commonly found in core facilities, whereas execution of some highly multiplexed approaches remains confined to a relatively small number of labs. The commercialization of many key assays is making their implementation more straightforward, although, in some cases, the high cost of consumables may remain an important consideration, and optimization for a particular tissue of interest remains a critical step. Despite these considerations, the yield of highly multiplexed profiling techniques is immense, allowing us to generate increasingly comprehensive pictures of tissue composition and reshaping our understanding of what distinguishes cells from one another and the biological networks that control these distinctions. The rapid growth of new and improved technologies in this arena is continuing to push toward higher throughput and more accurate and accessible tools that detect greater and greater numbers of features.

Goal 2: Identification of New Features or Cell Types

The majority of strategies described above delineate cells according to a limited set of candidate features, necessarily introducing the investigator's preconceptions about which features might be important to examine. Exciting developments over the past few years, particularly in single-cell sequencing technology, now allow profiling cells from diverse tissues in an unbiased manner. These strategies are being used to identify new cell types and to annotate known cell types with new constellations of markers.

mRNA sequencing from single cells (scRNA-seq) has become the leading technology for molecular profiling of the cellular composition of organs and organisms (Cao et al., 2017; Fincher et al., 2018; Karaiskos et al., 2017; Plass et al., 2018; Regev et al., 2017; Tabula Muris Consortium et al., 2018; Figure 2Avi). In brief, the scRNA-seq workflow involves isolating single cells and assigning each a unique barcode so that mRNA from many cells can be pooled for sequencing and subsequently re-assigned to its cell of origin (Hashimshony et al., 2012; Jaitin et al., 2014; Klein et al., 2015; Macosko et al., 2015; Ramsköld et al., 2012; Tang et al., 2009; reviewed in Ziegenhain et al., 2018). Cells can then be grouped based on the similarity of their transcriptomes through unsupervised clustering (reviewed in Kiselev et al., 2019). These analyses are revealing heterogeneity within

populations previously assumed to be homogeneous; for example, the spatial variation of diverse cell types in the intestinal epithelium, including tuft cells, enteroendocrine cells, and enterocytes (Beumer et al., 2018; Glass et al., 2017; Haber et al., 2017; Herring et al., 2018; Moor et al., 2018). They are also uncovering new and rare cell types (Grün et al., 2015; Jindal et al., 2018), such as the pulmonary ionocyte in the mammalian airway (Montoro et al., 2018; Plasschaert et al., 2018). Efforts are ongoing to reduce the cost of scRNA-seq approaches through multiplexed barcodes, increasing the accessibility, throughput, and potential applications of these approaches for mechanistic studies (Cao et al., 2017; Datlinger et al., 2019; Gehring et al., 2020; Kang et al., 2018; McGinnis et al., 2019; Rosenberg et al., 2018; Stoeckius et al., 2018).

Several important considerations exist for transcriptome-centric approaches. First, these analyses report not only on stable cell types but also on the transitions of cell types through states; for example, transiting through the cell cycle or maturation or activation states of immune cells (Jaitin et al., 2014; Shalek et al., 2013). Although, in some cases, capturing these state transitions may be desirable, in other cases, their effects may mask biological signals of interest. For example, heterogeneity between cells in different cell cycle stages can confound inference of developmental trajectories so that, in some cases, it is preferable to regress out the effect of the cell cycle from the dataset (Buettner et al., 2015; Vento-Tormo et al., 2018; discussed further in Luecken and Theis, 2019). These analyses also require dissociation of cells from the tissue, which can alter transcriptional profiles (van den Brink et al., 2017), although this effect can be mitigated by treatment with the transcriptional inhibitor actinomycin D (Act-seq; Wu et al., 2017). In addition, some cells are more sensitive to the dissociation process than others, which can introduce bias in the cells that are recovered. This dissociation bias can be reduced by sequencing RNA from individual nuclei rather than cells (single-nucleus RNA sequencing [snRNA-seq or sNuc-seq]; Grindberg et al., 2013; Habib et al., 2016; Koenitzer et al., 2020; Lake et al., 2016).

Importantly, the cellular dissociation required for many scRNA-seq approaches results in loss of valuable information regarding the spatial context of the cells. To overcome this problem, numerous strategies have been developed to combine transcriptional profiling with spatial information. One possibility is to infer the position of scRNA-seq profiles based on their expression of key landmark genes for which the spatial position is known from ISH atlases (Achim et al., 2015; Karaiskos et al., 2017; Satija et al., 2015). Alternatively, cells from defined positions can be isolated by laser capture microdissection before sequencing (Baccin et al., 2020; Moor et al., 2018; Zechel et al., 2014), by photoactivation and cell sorting (NICHE-seq; Medaglia et al., 2017), or by using a photo-uncaging system to hybridize DNA oligonucleotides to cells in illuminated regions (ZipSeq; Hu et al., 2020). Recent technologies have also employed more gentle tissue dissociation that preserves cell conjugates to identify interacting cells (Boisset et al., 2018; Giladi et al., 2020; Halpern et al., 2018).

A subset of approaches combines transcriptional profiles with spatial information by maintaining tissue architecture during profiling. For example, short sequences can be sequenced directly in cells fixed on a microscope slide. This approach, called fluorescence *in situ* sequencing (FISSEQ), uses the same *in situ* cDNA synthesis and rolling circle amplification principles described above for multiplexed mRNA detection. However, instead of sequencing user-defined barcodes, this approach sequences approximately 30 bp of the transcript itself, allowing unbiased determination of the identity of each amplicon (Lee et al., 2014; Figure 2Aix'''). An alternative suite of approaches introduces spatial barcodes during the scRNA-seq sample preparation so that RNAs can be pooled for sequencing but subsequently mapped back to their coordinates. For example, INSTA-seq (Fürth et al., 2019) uses a sequencing-by-ligation approach similar to FISSEQ with reduced imaging cycles to detect 12-bp barcodes from each amplicon *in situ* before next-generation sequencing. The barcode then allows the reads to be mapped back to the amplicon's position in the cell. Alternative approaches introduce a positional barcode into each cDNA by arraying RNA-capture oligonucleotides with a unique barcode at each position (Figure 2Avi). The first application of this approach allowed discrimination between RNAs with ~100 μm resolution (Ståhl et al., 2016). Recent developments have improved the resolution of such approaches, first to 10 μm (Slide-seq; Rodriques et al., 2019; Stickels et al., 2020) and subsequently to 2 μm (high-definition spatial transcriptomics [HDST]; Vickovic et al., 2019). Finally, the development of multiplexed single molecule FISH approaches to detect ~10,000 genes described above (MERFISH coupled with expansion microscopy [Xia et al., 2019] and SeqFISH+ [Eng et al., 2019]) open up the possibility that multiplexed single-molecule FISH, which previously required upfront selection of candidate genes, can be used for unbiased transcriptome-wide profiling with spatial resolution.

Approaches for spatial transcriptomics are evolving rapidly, and although tradeoffs exist between approaches, new technologies are rapidly overcoming limitations. Positional barcoding approaches have recently been commercialized (now marketed as Visium), rendering these approaches particularly accessible. However, they have lower detection efficiencies compared with FISH-based detection approaches such as MERFISH and SeqFISH. An added benefit of optical approaches (FISH and *in situ* sequencing) is that they provide information regarding subcellular localization of mRNAs, which plays important roles in diverse cellular functions (Jung et al., 2014; Lécuyer et al., 2007; Moor et al., 2017). Importantly, FISH-based approaches still require pre-designed oligonucleotides and, therefore, do not facilitate identification of unexpected transcript variants, including single-nucleotide variants that can be detected by sequencing-based approaches.

Although transcriptome-centric strategies currently dominate unbiased cell categorization efforts, other -omics-level profiling can facilitate discrimination between cells and add more layers to cellular definitions. Recent work has reported label-free proteomics profiling from single cells by ultrasensitive mass spectrometry (Virant-Klun et al., 2016; Zhu et al., 2018; Figure 2Aiii). This allows clustering of cells, assignment of new proteins associated with specific cell types, identification of heterogeneity within populations, and ordering of cells along a developmental trajectory (Specht et al., 2019; Zhu et al., 2019), although these approaches are still in their infancy.

A particular focus of recent work has been profiling of chromatin structure and composition (Figure 2B). A subset of bulk approaches for genome-wide profiling of DNA modifications, histone modifications, protein-DNA interactions, and chromatin accessibility have been modified for use in single cells ((Figure 2B; reviewed in Ludwig and Bintu, 2019; Shema et al., 2019), facilitating their use for cell type classification. For example, DNA methylation signatures can distinguish cell types in the mammalian cortex (Luo et al., 2017; Mulqueen et al., 2018) as well as identify sister cells in the four-cell mouse embryo (Mooijman et al., 2016). Similarly, single-cell profiling of chromatin modifications or protein-DNA interactions (Figure 2B) can discriminate cellular subpopulations (Grosselin et al., 2019; Kaya-Okur et al., 2019; Rotem et al., 2015; Wang et al., 2019). A particularly powerful approach for profiling cell types is genome-wide profiling of chromatin accessibility; for example, nucleosome positioning through DNase digestion (scDNAse-seq; Jin et al., 2015) or micrococcal nuclease digestion (MNase-seq; Lai et al., 2018), or exposed DNA based on the preferential integration of transposons (single-cell ATAC-seq and single-cell combinatorial indexing ATAC-seq; Buenrostro et al., 2018; Chen et al., 2018; Cusanovich et al., 2018a, 2018b). These studies highlight particular strengths of epigenomic profiling for cell type characterization, including the capacity to identify distal regulatory elements shaping gene expression and detect epigenetic changes that precede changes in gene expression (Inoue et al., 2019; Ziffra et al., 2019) and which may, for example, be suggestive of priming for differentiation toward distinct lineages (Buenrostro et al., 2018; Lai et al., 2018).

Combined profiling of genomic, epigenomic, transcriptomic, and proteomic features can further refine cellular delineations and reveal underlying regulatory relationships (Figure 2C). For example, numerous approaches layer additional molecular measurements on top of scRNA-seq data, combining transcriptomic profiling with genome-wide profiling of copy number variants, DNA methylation, and chromatin accessibility as well as measurements of candidate proteins (Figure 2C; reviewed in Zhu et al., 2020). These approaches can map additional molecular layers onto scRNA-seq-defined cell types and, in some cases, distinguish cellular sub-populations beyond those identified from transcriptomic profiling alone (Stoeckius et al., 2017; Ziffra et al., 2019). In addition to simultaneous measurements of distinct molecular features from the same cell or the same pool of cells processed in parallel, new algorithms are facilitating integration of discrete transcriptomic, epigenomic, and targeted proteomics datasets (Stuart et al., 2019; Welch et al., 2019). Together, these approaches move toward a more complete picture of cell identity and its underlying regulation while further amplifying the challenge of determining the extent to which additional heterogeneity identified at each layer connects to variability at the functional level.

Goal 3: Define Cellular Relationships

3a: Live Microscopy. A central component of a cell's identity is its position in the lineage hierarchy, meaning the identities of its mother and/or daughter cells. Live microscopy (Figure 3A) can reveal the ground truth of these mother-daughter relationships by direct observation, in contrast to approaches that infer cellular relationships from snapshots of cells at discrete time points. Live imaging approaches can capture cell divisions, cell movements, cell death, and changes in morphology (Figure 1) and allow continuous observation of specific cells over time to identify heterogeneities in behavior within a population, such as rates of differentiation. Advances in *in vitro* cell culture systems as well as imaging technologies and data analysis pipelines are rapidly increasing the resolution, time frames, and throughput of the assessment of the dynamics of cellular relationships.

The power of direct continuous observation for defining cellular hierarchies is exemplified by the pioneering work of Charles Whitman (Whitman, 1887), E.B. Wilson (Wilson, 1892), Edward Conklin (Conklin, 1897), and others in the early embryos of marine invertebrates and by John Sulston's studies (Sulston et al., 1983) that defined the complete lineage tree of *Caenorhabditis elegans*. In the stem cell field, live-imaging approaches for defining cell lineage have been empowered by the development of *in vitro* stem cell culture systems that recapitulate aspects of *in vivo* division and differentiation patterns in two dimensions as well as in three-dimensional culture systems such as organoids or explants. For example, mammalian neural stem and progenitor cell cultures derived from rodent embryos and adults as well as human fetal brain can be imaged by phase contrast microscopy every few minutes over the course of 1 or 2 weeks, and progeny fates can be defined by morphology and post-imaging immunostaining. This approach has allowed direct assessment of asymmetric versus symmetric divisions and the construction of lineage trees, as well as identification of the relative timing of differentiation events (for example, generation of neurons and glial cells; Costa et al., 2011; Piltti et al., 2018; Qian et al., 1998, 2000; Ravin et al., 2008; Winter et al., 2015). In the hematopoietic system, live imaging and tracking of embryonic stem cell-derived cells and primary hematopoietic progenitors have clarified the generation of blood cells from embryonic endothelial cells (Eilken et al., 2009) and the instructive role of cytokines in generating monocytic or granulocytic cells from bipotent cells (Rieger et al., 2009). Similar approaches have also revealed heterogeneities in the differentiation of embryonic stem cells exposed to bulk signals (Brown et al., 2017a) as well as the ability of local signals to instruct cell division orientation and gene expression (Habib et al., 2013). These studies highlight the power of *in vitro* systems to identify changes in cellular properties over time and dissect mechanisms underlying cell fate choices by facilitating direct and rapid perturbations. Moreover, understanding cellular behavior in culture provides critical information for therapeutic applications that may require expansion of cells in culture before transplantation.

Live imaging can also be used for dynamic assessment of cellular behaviors and relationships in living organisms using a variety of approaches to render the tissue of interest optically accessible. In some cases, internal organs can be surgically exposed (Ewald et al., 2011); for example, revealing immune cell migration patterns in the liver and spleen (Egen et al., 2008; Swirski et al., 2009), but these experiments are generally terminal. Implantation of a transparent window can facilitate repeated imaging sessions (Sandison, 1924). Dorsal skinfold chambers (Algire and Legallais, 1949) allow visualization of xenograft cell behaviors (Brown et al., 2001), such as cell division and

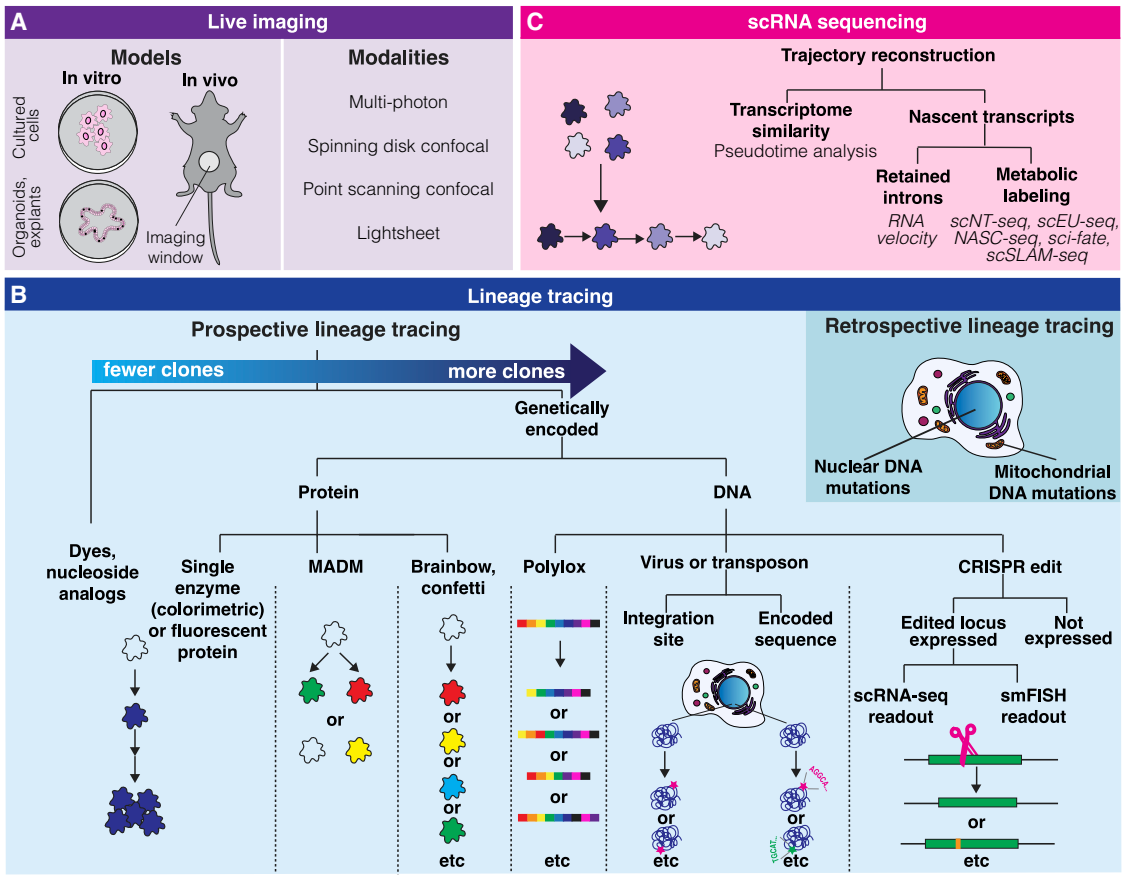

Figure 3. Strategies to Define Cellular Relationships
(A) Models and modalities to facilitate live imaging of cellular relationships.
(B) Lineage tracing approaches, grouped according to the kind of reporter used by the system.
(C) Approaches for defining cellular relationships with scRNA-seq.

therapeutic response (Orth et al., 2011). Alternatively, windows can be implanted over the organ of interest (Alieva et al., 2014). This approach has been applied particularly with mammary imaging windows and abdominal imaging windows; for example, to track cell behaviors and stem cell dynamics in healthy tissues (Ritsma et al., 2014; Scheele et al., 2017) as well as the cellular dynamics of tumor growth and metastasis (Kedrin et al., 2008; Ritsma et al., 2012; Sobolik et al., 2016). In other cases, structures can be imaged directly without surgical interventions, and sites of interest can be revisited over multiple imaging sessions using anatomical markers or tattoos as references. For example, the calvarium (skull bones) of the mouse is sufficiently transparent to visualize behaviors of hematopoietic stem/progenitor cells or leukemic cells (Adams et al., 2009; Christodoulou et al., 2020; Colmone et al., 2008; Lo Celso et al., 2009; Mazo et al., 1998; Sipkins et al., 2005). The accessibility of the skin has rendered this organ particularly powerful to uncover cellular dynamics through optical approaches such as live imaging and targeted laser ablation to disrupt specific cells (Rompolas et al., 2012, 2013). Intravital imaging studies have generated a wealth of information regarding the cellular behaviors required for tissue function (Marsh et al., 2018) and revealed heterogeneous behaviors within cell populations based on differences in their local environment, as we discuss further in Assessing Cell Function: Modulation of Cellular Contributions by Tissue Context (Mesa et al., 2018; Ritsma et al., 2014; Rompolas et al., 2013, 2016).

Live-imaging analyses of cellular relationships are facilitated by diverse microscopy techniques coupled with technological advances for automated cell segmentation, tracking, and lineage reconstruction (Amat et al., 2014; Bao et al., 2006; Du et al., 2014; Faure et al., 2016; Mace et al., 2013; McDole et al., 2018; Ulman et al., 2017; Wan et al., 2019; Wolff et al., 2018). Broadly, a tradeoff exists between the physiological complexity of the system and technical complexity of the imaging experiment, with considerations including resolution, speed, signal to noise, cost, phototoxicity, and, increasingly, the computational challenges of the data analysis (reviewed in Combs and Shroff, 2017; Thorn, 2016). Conventional wide-field microscopy has proved useful for imaging sparsely labeled cells in transparent organisms, such as developing zebrafish (Kimmel et al., 1990; Woo and Fraser, 1995). However, confocal microscopy (Minsky, 1961), which rejects out-of-focus light through use of a pinhole in front of the detector, has been particularly valuable for tissue imaging because it allows thick samples to be imaged in discrete optical sections.

A major challenge for tracing cellular hierarchies in living 3D tissues and organisms is that imaging illumination can damage the system so that the act of observing cellular behavior can alter it (Magidson and Khodjakov, 2013). Conventional wide-field and confocal microscopes illuminate fluorophores outside of the imaging focal plane, causing photodamage to regions that do not participate in generating the final image. In contrast, light-sheet fluorescence microscopy illuminates a single plane within the object at a given time (Huisken et al., 2004), allowing low-photodamage optical sectioning. Broadly, light-sheet fluorescence microscopes use a cylindrical lens (Huisken et al., 2004), digital scanning laser beam (Keller et al., 2008) or Bessel beam (Planchon et al., 2011) to form a sheet that illuminates only a thin volume of the sample. Moving the specimen through the light sheet or scanning the sheet over the sample allows the full sample volume to be imaged. These approaches allow visualization of cell divisions and movements with high temporal and spatial resolution in developing organisms (Huisken et al., 2004; Keller et al., 2008; Krzic et al., 2012; McDole et al., 2018; Tomer et al., 2012; Udan et al., 2014; Wolff et al., 2018; Wu et al., 2013) and organoids (McKinley et al., 2018; Serra et al., 2019). Finally, efforts are underway to further reduce illumination requirements on conventional microscopes using deep learning-based approaches to improve the signal-to-noise ratio of images collected under low-light conditions (Fang et al., 2019; Weigert et al., 2018).

When imaging tissue, variations in refractive index between the sample and its medium and between different objects in the sample lead to optical aberrations that distort the image. Although efforts are underway to resolve medium-sample refractive index mismatches (Boothe et al., 2017), light scattering and absorbance from cellular components within the tissue remain major obstacles. For fixed tissue, clearing approaches that seek to equilibrate the refractive index throughout the sample can reduce scattering and allow high-resolution volumetric imaging (reviewed in Richardson and Lichtman, 2015). For living tissues, one possibility to circumvent this issue is to acquire images of the specimen from multiple angles (multiview imaging). This can be achieved by rotating the sample or by imaging from multiple positions simultaneously using additional objectives (Chhetri et al., 2015; Krzic et al., 2012; Royer et al., 2016; Tomer et al., 2012; Wu et al., 2013). These different views can then be registered computationally (Preibisch et al., 2010). Alternatively, adaptive imaging or adaptive optics approaches can detect optical aberrations and apply corrections to compensate for them (Ji, 2017; Liu et al., 2018; Royer et al., 2016; Wilding et al., 2016). This allows long-term imaging of systems that change their optical properties over time; for example, imaging the development of whole mouse embryos from embryonic day 6.5 (E6.5) to E8.5 (McDole et al., 2018). Greater tissue penetration can be achieved by using longer wavelengths, which, broadly, scatter less. For example, use of a far-red fluorescent reporter allows for light-sheet imaging of the developing mouse heart 600 μm deep within the embryo (McDole et al., 2018). The low-scattering nature of long-wavelength light also contributes to the capacity of two-photon microscopy to improve tissue penetration (Denk et al., 1990). In two-photon microscopy, a fluorophore is excited by absorbing two low-energy photons essentially simultaneously. Because the likelihood of two photons hitting the fluorophore rapidly falls off away from the focal point, two-photon microscopy minimizes out-of-focus fluorescence and generates extremely low background. The improved tissue penetration of two-photon imaging has made it particularly well suited for intravital imaging approaches. Together, these approaches provide a rapidly expanding toolkit for assessment of cellular relationships through direct observation.

3b: Lineage Tracing. Lineage tracing—using a heritable mark to track progeny of cells of interest—can facilitate identification of cellular relationships through live imaging or in fixed or dissociated tissues. Early lineage tracing studies took advantage of natural variations in pigmentation (Conklin, 1905; Rawles, 1948), gross chromosomal markers (Ford et al., 1956; Wu et al., 1968), or features such as heterochromatin distribution (Le Douarin, 1980). Alternatively, dyes can be applied or injected (Serbedzija et al., 1989; Vogt, 1929), or marker transgenes can be integrated into the genome by viral transduction (Price et al., 1987; Dick et al., 1985; Keller et al., 1985). Current applications focus particularly on tracing cellular progeny using endogenous or induced genetic variants (Figure 3B).

To identify the progeny of cells expressing a particular gene or small subset of genes, site-specific genetic recombination can be used to drive expression of a reporter gene, such as a fluorescent protein or enzyme, in the cells of interest and their progeny (reviewed in Hsu, 2015; Kretzschmar and Watt, 2012). These experiments commonly use Cre recombinase expressed under control of a cell-type-specific promoter, which catalyzes recombination at DNA recognition motifs called loxP sites to drive reporter expression. Recombination can be induced at a specific time point using doxycycline-inducible or tamoxifen-inducible Cre systems, with the caveat that high doses of tamoxifen have been found to affect the mammary gland (Rios et al., 2014; Shehata et al., 2014), pancreas (Ahn et al., 2019), intestine (Zhu et al., 2013), and stomach (Huh et al., 2010). In addition to Cre/lox, alternative pairs of recombinases and recombination sites include Flippase/Flippase Recognition Target (Flp/FRT) and Dre/Rox. This variety of recombination approaches can be used to simultaneously track multiple cell types and their progeny. In addition, they allow intersectional methods to mark a cell type identified by a unique combination of genes rather than a single gene by creating a logical AND gate, in which two genes must be expressed to drive marker expression (Hermann et al., 2014; Madisen et al., 2015). An AND gate can also be created by splitting Cre into two parts under the control of different promoters so that they can only form a functional Cre and drive recombination in cells in which both promoters are expressed (Casanova et al., 2003; Xu et al., 2007). Split Cre approaches can also be combined with domains that dimerize in the presence of defined wavelengths of light, allowing activation of Cre recombination in cells selected microscopically (Meador et al., 2019; Taslimi et al., 2016).

A variety of reporters are available that can mark all cells in the population uniformly upon recombination; alternatively, reporters that generate different marks within a population of cells allow progeny from different cells within the population to be distinguished (Figure 3B). For example, the mosaic analysis with double markers (MADM) system can mark sibling cells with distinct fluorescent proteins (Zong et al., 2005). In Brainbow or confetti reporters, recombination assigns one of many fluorescent proteins

to each cell at random (Livet et al., 2007; Snippert et al., 2010), The capacity to generate distinct fluorescent marks within the population allows many different cells expressing a common gene to be readily distinguished from one another; for example, to identify, track, and morphologically characterize them microscopically in complex environments (Currie et al., 2016; Livet et al., 2007) as well as to determine how cells derived from a common progenitor are distributed within a tissue (McKinley et al., 2018; Pan et al., 2013). Crucially, the capacity to track multiple independent clones can reveal whether progenitor cells within a population exhibit different potential (Ghigo et al., 2013; Rinkevich et al., 2011; Snippert et al., 2010). The resolution of such clonal lineage analysis improves with an increasing number of potential marks; the potential diversity of labels can be increased by replacing fluorescent reporters with DNA sequences, as in the *Polylox* reporter, which can generate a maximum of 1.8 million unique DNA barcodes in Cre-expressing cells (Pei et al., 2017). Such DNA-based tools facilitate high-resolution lineage tracing of a large number of clones compared with fluorescent proteins, albeit with loss of spatial and morphological information when dissociation is required to recover the barcode.

Numerous strategies are available to generate highly variable DNA sequences in cells of interest and their progeny. One approach uses a library of DNA barcodes to mark cells, which can be introduced into the cells by viral transduction (Walsh and Cepko, 1992; Lu et al., 2011; Schepers et al., 2008), zinc-finger-mediated homologous recombination (Porter et al., 2014), or transposition (TracerSeq; Wagner et al., 2018). If the barcode is transcribed, then the clonal relationships can be integrated with transcriptomic profiling by scRNA-seq (Biddy et al., 2018; Wagner et al., 2018; Weinreb et al., 2020; Yao et al., 2017). Alternatively, as the virus integrates quasi-randomly into the genome, the insertion site can function as the barcode. This approach has been powerful for studies of the human hematopoietic system, for which cells can be barcoded *ex vivo* and transplanted to trace clonal dynamics during repopulation *in vivo* in mice (Guenechea et al., 2001; Lu et al., 2011; McKenzie et al., 2006; Nolta et al., 1996) as well as non-human primates (Schmidt et al., 2002; Yu et al., 2018). Moreover, use of viral vectors for gene therapy has allowed longitudinal profiling of insertion sites following transplantation of gene-corrected hematopoietic stem/progenitor cells into human patients, particularly as a test for clonal dominance events induced by the insertion (Aiuti et al., 2013; Biffi et al., 2013). Importantly, the transplantation process to introduce virus-barcoded cells *in vivo* may affect cellular functions. For example, a mouse model that mobilizes a transposon to generate insertion-site barcodes without transplantation allowed profiling of steady-state hematopoiesis (Sun et al., 2014) and revealed significant differences from the transplantation hematopoietic hierarchy (Rodriguez-Fraticelli et al., 2018; reviewed in Baron and van Oudenaarden, 2019). We discuss modulation of cell function by transplantation further in Assessing cell function: Modulation of Cellular Contributions by Tissue Context.

Dynamic editing of a target locus is increasingly used to generate diverse DNA-based barcodes for lineage tracing. The majority of these systems use CRISPR/Cas9 mutagenesis, in which Cas9 is directed to cut at a specific sequence in the genome based on complementarity with a short, user-supplied RNA sequence called a single guide RNA (sgRNA). This results in genetic lesions in the selected regions through error-prone repair of double-strand breaks (reviewed in McKenna and Gagnon, 2019). These approaches target Cas9 to cut within synthetic arrays, as in GESTALT (McKenna et al., 2016), scGESTALT (Raj et al., 2018), MEMOIR (Frieda et al., 2017), CARLIN (Bowling et al., 2019), Zombie (Askary et al., 2020), and others (Chan et al., 2019). Alternatively, Cas9 can cut within fluorescent proteins, as in the ScarTrace method (Alemany et al., 2018; Junker et al., 2017), LINNAEUS (Spanjaard et al., 2018), and others (Schmidt et al., 2017). Further, a subset of approaches direct cuts within the sequence encoding the sgRNA itself, in the case of the MARC1 mouse (Kalhor et al., 2018) and mSCRIBE (Perli et al., 2016). The mutations are read out by sequencing the target locus (Alemany et al., 2018; Junker et al., 2017; McKenna et al., 2016; Schmidt et al., 2017), or, if the barcode is transcribed, by scRNA sequencing (Alemany et al., 2018; Bowling et al., 2019; Chan et al., 2019; Raj et al., 2018; Spanjaard et al., 2018) or smFISH (Frieda et al., 2017; Askary et al., 2020). Recent work has also used phage integrases as an alternative approach to CRISPR to generate high-diversity dynamic barcodes that can be read out by smFISH (intMEMOIR; Chow et al., 2020). The high diversity of DNA-based lineage tracing approaches makes them amenable for simultaneous lineage tracing from a wide variety of different cell types. This largely unbiased approach not only generates lineage hierarchies with much higher throughput but is also revealing intriguing circumstances in which cells from different lineages converge on a given transcriptional signature, suggestive of convergent differentiation (Chan et al., 2019; Wagner et al., 2018). Thus, these unbiased approaches are revealing that the synthesis of cell lineage and cell features can refine models for cell identity and differentiation trajectories.

Finally, in addition to introduced DNA edits, lineage can also be traced retrospectively using spontaneous somatic mutations (Behjati et al., 2014; Frumkin et al., 2005), such as long interspersed nuclear element 1 (LINE-1) retrotransposition events, copy number variants, single-nucleotide variants, and microsatellite growth or shrinkage. In addition to mutations in nuclear DNA, it is possible to use mutations in mitochondrial DNA, which are highly enriched in ATAC-seq libraries, for simultaneous profiling of lineage relationships and chromatin accessibility (Ludwig et al., 2019; Xu et al., 2019; Figure 3B). These retrospective lineage tracing approaches allow identification of cellular hierarchies in systems not amenable to directed genetic modification and have therefore provided significant insights into lineage hierarchies for human tissues such as the brain, blood, and embryo (Biezuner et al., 2016; Cai et al., 2014; Evrony et al., 2015; Ju et al., 2017; Lee-Six et al., 2018; Lodato et al., 2015; Osorio et al., 2018).

3c: Single-Cell Transcriptomics. Even without a lineage-tracing component, scRNA-seq technologies can provide information about the relationships between cells (Figure 3C; reviewed in Lederer and La Manno, 2020). Because existing scRNA-seq pipelines lyse or fix cells to define transcriptomes, it is not possible to track changes over time on a per-cell level. However, many cell types at different stages of differentiation are present within a tissue at a given time so that, when the population is considered as a whole, a static snapshot can encapsulate all of the steps along the differentiation trajectories of a tissue (for example,

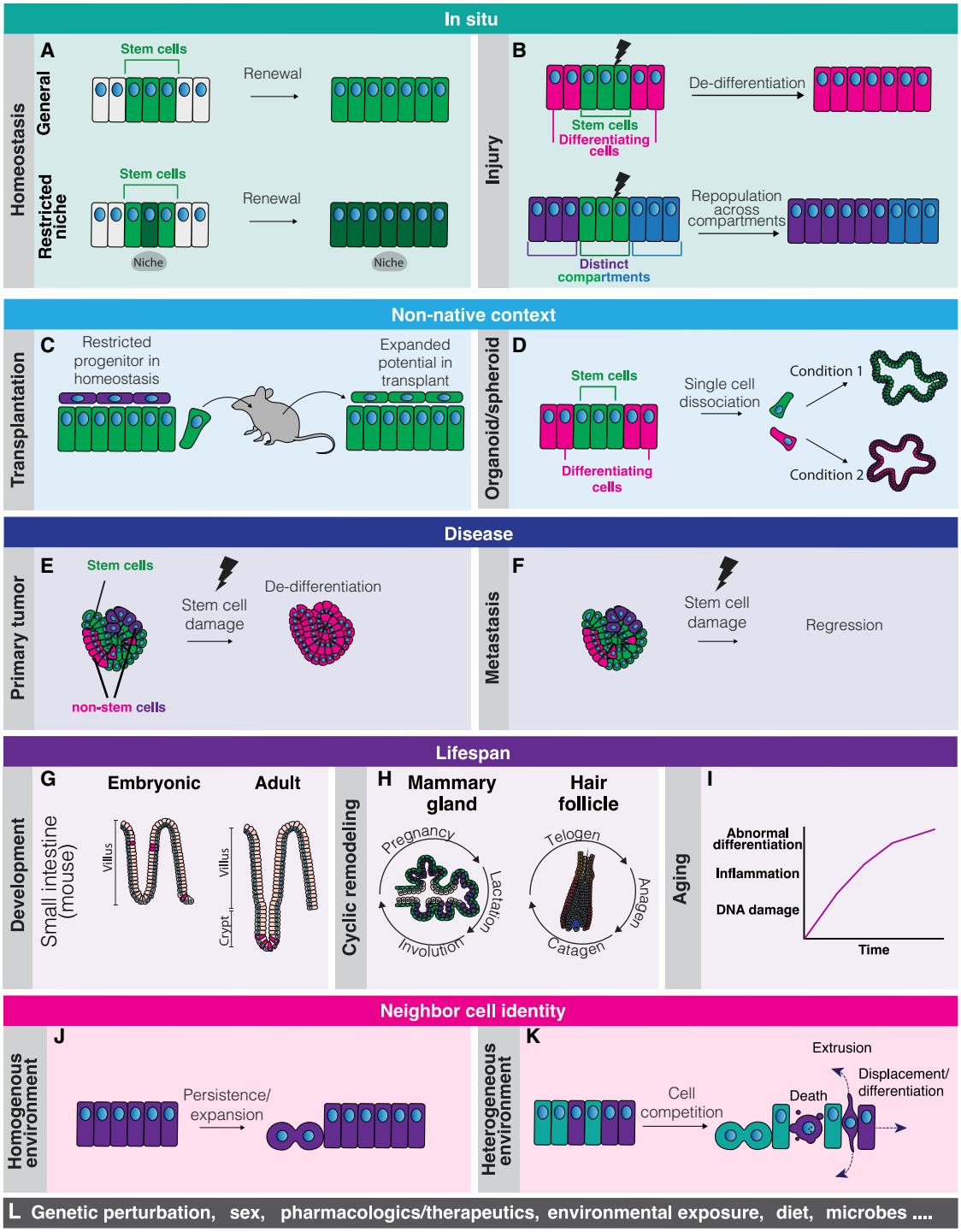

Figure 4. Cellular Functions Vary with Context

(A) In homeostasis, canonical stem cells (green) give rise to all cells of the epithelium, including new stem cells. In some tissues, stem cells in close proximity to niche factors (dark green) expand preferentially over more peripheral stem cells (light green).

(B–D) Cells with diverse lineage histories can exhibit stem-like functions in different physiological and experimental contexts. Experimental and pathological alterations including injury (B), transplantation (C), and *ex vivo* culture (D) can expand the repopulation capacity of a cell type, endowing cells other than the canonical stem cell with the capacity to repopulate tissue (pink cells) or allowing cells to repopulate across previously non-permissive lineage boundaries.

(E and F) Cell function and contribution can vary with disease stage. For example, primary colorectal tumors can be maintained even if the stem cells are ablated (E). In contrast, liver metastases derived from colorectal cancers regress when the stem cells are ablated (F).

(legend continued on next page)

Haber et al., 2017; Halpern et al., 2017)) or whole organism (Plass et al., 2018; Siebert et al., 2019). Samples can also be taken from multiple different time points, allowing characterization of differentiation trajectories across development of entire embryos (Briggs et al., 2018; Farrell et al., 2018; Wagner et al., 2018). Differentiation trajectories can be inferred from these snapshot data by pseudotime analysis, also known as trajectory inference, which orders cells based on similarity in their gene expression (Haghverdi et al., 2016; Trapnell et al., 2014). Over 70 trajectory inference tools have been developed, which are reviewed and evaluated elsewhere (Saelens et al., 2019; Tritschler et al., 2019; Weinreb et al., 2018). Importantly, pseudotime analysis infers ordering of cells according to their relative progress through a biological process but does not provide information about the actual duration of events. Recent efforts have sought to couple real-time information with transcriptional ordering. For example, a recent study used a bifluorescent reporter comprised of two fluorophores with different maturation kinetics and half-lives as a cellular timer that positions transcriptional profiles relative to real time (Gehart et al., 2019).

New technologies and approaches are moving beyond inferred differentiation trajectories to direct measurements of dynamics from single-cell transcriptomics data. Pioneering work revealed that scRNA-seq data not only report a cell's gene expression levels at the time point of the experiment but also contain information regarding a cell's immediate future by incidentally capturing newly transcribed, unspliced precursor mRNAs (La Manno et al., 2018). The relative amounts of nascent RNA and mature mRNA are reflective of how gene expression is changing in the cell. When a gene has been recently activated, there are more unspliced transcripts than spliced; conversely, when a gene has been recently repressed, spliced transcripts persist, but unspliced transcripts decrease. Thus, measurements of spliced and unspliced transcripts reveal the rate of change of mRNA (RNA velocity) and, therefore, predict future mRNA abundance and forthcoming transcriptional states. Ongoing work is extending this framework; for example encompassing multi-omics data (Gorin et al., 2019) and reconstructing trajectories from sparse velocity information (Qiu et al., 2019). Alternatively, because mRNAs are transcribed in the nucleus and exported to the cytoplasm upon maturation, relative nuclear and cytoplasmic mRNA abundance, measured by FISH, can also predict future transcriptional states *in situ* (Xia et al., 2019). Finally, although RNA velocity takes advantage of serendipitously captured nascent RNA, metabolic labeling approaches in which nascent RNA is labeled with 4-thiouridine (4sU) or 5-ethynyl-uridine (EU) have recently been coupled with scRNA-seq to facilitate direct assessment of future transcriptional profiles in single cells (Battich et al., 2020; Cao et al., 2020; Erhard et al., 2019; Hendriks et al., 2019; Qiu et al., 2019).

The toolkits to detect, identify, and analyze diverse cellular features and functions are expanding at breakneck speed. These approaches are continuing to push us toward a more precise mechanistic understanding of organ function during homeostasis.

Assessing Cell Function: Modulation of Cellular Contributions by Tissue Context

As the tools and strategies described above are applied to organs experiencing mutational, damage, and disease burdens, they are revealing that the connections between cellular features and functions are highly dependent on a cell's context. In this section, we discuss recent studies assessing cellular functions across diverse contexts. We first discuss how experimental or pathological alterations are reshaping our understanding of stem cell function. We then examine how cellular context can determine whether a cell expands within the tissue or is eliminated through competitive interactions. These studies are underscoring the limitations of viewing cell identity as a hardwired, intrinsic property and expand our understanding of the intricate relationships between cells and their microenvironment.

Functional Definitions of Epithelial Stem Cells

Many efforts to define cellular functions are focused on the identification and characterization of stem cells, which hold significant potential for therapeutic applications because of their capacity to expand and generate diverse cell types. Broadly, stem cells are defined by their capacity to maintain the stem cell population through self-renewal and to generate many distinct differentiated cell types. Initial models proposed that epithelial stem cells execute both functions with every division, generating one stem cell and one differentiating cell through asymmetric divisions (Potten, 1974). However, it has become clear that this paradigm does not hold in many epithelia. Pioneering work in the skin demonstrated that, in addition to asymmetric divisions yielding one proliferating cell and one committed cell, progenitors could also undergo symmetric divisions that generate two progenitors or two committed progeny (Clayton et al., 2007). Subsequent work in the male germline and intestine of mice and flies showed that stem cells in these tissues can be stochastically lost and replaced by new stem cells arising from symmetric division of their neighbors (de Navascués et al., 2012; Klein et al., 2010; Lopez-Garcia et al., 2010; Sheng and Matunis, 2011; Snippert et al., 2010). Later work has revealed similar principles at work in other solid tissues, including the esophagus (Doupé et al., 2012), oral mucosa (Jones et al., 2019), and epidermis (Rompolas et al., 2016). These experiments and others have focused the definition of epithelial stem cells on their ability to renew the stem cell population and generate differentiating cells of diverse lineages at the population level rather than at each division (Post and Clevers, 2019).

Although epithelial stem cells are broadly capable of generating progeny that adopt differentiating or stem cell fates, these decisions can be biased by local variations in the microenvironment, such as proximity to niche signals. For example, the mouse intestine is compartmentalized into invaginations called crypts, each containing numerous stem cells. Over time, these

(G) Cellular relationships can be altered between developing tissues and adult tissues. For example, in the mouse small intestine, during development all cells of the epithelium can become stem cells, whereas in the adult, stem cells are restricted to the base of crypts.
(H) A subset of tissues exhibit cyclic differences in cellular composition and function.
(I) Cells in many tissues, such as the skin, intestine, oral mucosa, and hematopoietic system, exhibit a variety of alterations with age.
(J and K) Cell competition can cause cells that can normally sustain and repopulate a tissue (J) to be selectively eliminated when combined with fitter neighbors (K).
(L) In addition to the contexts presented in (A)–(K), diverse additional sources of variability can alter cellular functions.

crypts become clonal, as progeny from one stem cell eventually take over the entire unit (Griffiths et al., 1988; Potten and Loeffler, 1990; Winton et al., 1988). Intravital imaging revealed that stem cells at the bases of intestinal crypts are approximately three times more likely to colonize the crypt than stem cells at more peripheral positions (Ritsma et al., 2014). Similarly, live-imaging of the hair follicle demonstrated that the position of a stem cell within the hair follicle niche correlates with stem cell fate (Rompolas et al., 2013). Intriguingly, recent work has demonstrated that adult stem cells in the intestine arise from cells that receive a positional advantage during reorganization of the tissue during development (Guiu et al., 2019). These data suggest that proximity to niche signals can allow a subset of cells to expand preferentially (Figure 4A). In addition, neighboring cell behavior can also modulate stem cell expansion. For example, recent live-imaging analysis of epidermal stem cell behavior revealed that epidermal stem cell division is triggered by neighboring stem cell differentiation (Mesa et al., 2018). Together, these studies suggest that microenvironmental signals can distinguish between apparently homogeneous stem cells, privileging the expansion of subsets of cells and generating functional heterogeneity within epithelial stem cell populations.

Functional Tests of Stemness

Extensive work has sought to characterize stem cells in diverse epithelia by identifying specific cellular features and associated markers that enrich for cells with the long-term potential to populate a tissue. As ongoing studies test stemness with new technologies and in new contexts, they are expanding our understanding of stem cell function and revealing limitations of previous definitions and experimental strategies. In particular, these studies have shown that repopulation potential can segregate with different cellular features depending on the physiological context or the technique used to assess it (Figures 4B–4D).

Transplantation is a long-standing approach to identify stem cell populations (Figure 4C). Trailblazing work in the 1950s demonstrated that transplanted material could repopulate the hematopoietic system following irradiation (Ford et al., 1956; Lorenz et al., 1951) and the mammary gland following fat pad clearing (Deome et al., 1959). Moreover, the engrafted material could be re-transplanted into secondary recipients, indicating long-term self-renewal capacity (Barnes et al., 1959; Daniel et al., 1968; Deome et al., 1959; Hoshino and Gardner, 1967). In the hematopoietic system, this approach demonstrated the existence of multipotent, self-renewing stem cells (Becker et al., 1963; Siminovitch et al., 1963; Till and McCulloch, 1961; Wu et al., 1968) that were subsequently prospectively isolated by their complement of cell surface markers (Spangrude et al., 1988). These early basic science discoveries have led to enormous clinical advances; hematopoietic stem cell transplantation has revolutionized the treatment of hematological disorders and malignancies (Appelbaum, 2007).

However, transplantation studies have also given rise to controversy over the potential of stem cell populations. In the mammary gland, transplantation studies demonstrated that increasingly refined cell populations and, eventually, single cells, could give rise to both of the major epithelial lineages of the mammary gland: luminal cells and basal myoepithelial cells (Kordon and Smith, 1998; Shackleton et al., 2006; Stingl et al., 2006). However, subsequent work suggested that the mammary gland is repopulated under physiological conditions by lineage-restricted progenitors (Van Keymeulen et al., 2011), suggesting that the broader multipotency observed in the transplant studies resulted from plasticity induced by the new cellular microenvironment. The extent to which the mammary gland is maintained by bi-potent stem cells or unipotent progenitors is still the subject of debate (reviewed in Lloyd-Lewis et al., 2017). Similarly, in the skin, LRIG1-positive cells can give rise to all epidermal lineages following grafting, but they exhibit more limited potential and contribute only to the interfollicular epidermis and sebaceous gland under steady-state conditions (Jensen et al., 2009). In addition to expanded potential when transplanted orthotopically into a damaged site, transplantation to an ectopic site can also expand cellular potential. One dramatic example of environment shaping function is that epithelial cells of the thymus can contribute to hair follicle lineages following grafting (Bonfanti et al., 2010).

These studies reveal that the interpretation of transplantation experiments aiming to characterize stem cells can be affected by introducing cells into a new microenvironment as well as through possible injuries and regenerative programs induced by the transplantation process. In contrast, the advancement of lineage tracing methods described above has provided a minimally invasive solution to assess stem cell potential *in situ*. Nonetheless, transplantation approaches continue to facilitate new discoveries in stem cell function in health and disease, particularly for genetically intractable systems such as humans (Shimokawa et al., 2017) and also provide important information about the potential of cells to contribute therapeutically to repair.

An alternative approach to assess stem cell function is to determine the capacity of a cell type to expand and generate diverse cell types in culture. For example, in some cases, single cells plated *in vitro* in extracellular matrices can give rise to organoids—stable, complex tissues with diverse cellular composition—indicating the self-renewal capability and multilineage potential of the cells (Figure 4D). Based on early work showing that 3D culture can generate structures with functional properties and cell composition resembling the *in vivo* mammary gland (Barcellos-Hoff et al., 1989; Lee et al., 1985), the formation of mammary gland cultures capable of propagation in culture, called mammospheres (Dontu et al., 2003), was adopted as a strategy to isolate mammary gland stem cells (Liao et al., 2007). The subsequent identification of organoid-forming capacity in single intestinal stem cells (Sato et al., 2009) has led to the now widely used intestinal organoid model. This approach has been subsequently expanded to tissues including the stomach (Barker et al., 2010; Stange et al., 2013), liver (Hu et al., 2018; Huch et al., 2013), airway (Rock et al., 2009), and tongue (Hisha et al., 2013; Ren et al., 2014). Importantly, because organoids are minimal systems removed from their native context, they require supplementation with growth factors to support their growth. The combination of additives present in the medium has a significant effect on organoid formation capacity. For example, depending on the culture conditions, intestinal organoid formation is either restricted to cells expressing the gene *Lgr5*, reflective of their unique multilineage potential under homeostasis, or expanded to non-LGR5-positive cells (Castillo-Azofeifa et al., 2019; Serra et al., 2019; van Es et al., 2012). Thus, the process of generating organoids can also uncover non-homeostatic potential, and when assessing stemness through organoid-forming

potential, it is important to consider the extent to which the culture conditions reflect the *in vivo* microenvironment.

Modulation of Stem Cell Behavior by Damage

In addition to experimental manipulations, tissue damage can also cause stem cell populations to expand their potential or cause new cell populations to acquire stem-like properties (Figure 4B). For example, in the skin, stem cells from distinct compartments can mobilize toward wounds to repair damaged tissue, in some cases occupying new niches and adopting the stem cell function associated with their new position (Hoeck et al., 2017; Ito et al., 2005; Levy et al., 2007; Page et al., 2013; Rompolas et al., 2013; Snippert et al., 2010). Additional work has revealed that cells can also cross lineage boundaries following damage in tissues of the intestine, stomach, teeth, and lung (Ayyaz et al., 2019; Castillo-Azofeifa et al., 2019; Jadhav et al., 2017; Sharir et al., 2019; Tata et al., 2013; Tian et al., 2011; van Es et al., 2012; Yui et al., 2018; reviewed in Burclaff and Mills, 2018; de Sousa e Melo and de Sauvage, 2019; Tata and Rajagopal, 2017). These results call to mind classical experiments in *C. elegans* that demonstrated that cells could compensate for cell types lost by laser ablation (Kimble, 1981; Sulston and White, 1980).

Extensive work in both flies and mammals has revealed that cells that have already made fate commitments can revert into stem cells in response to stresses, essentially reversing the traditional lineage hierarchy in homeostasis (Tata et al., 2013; Tian et al., 2011; Brawley and Matunis, 2004; Kai and Spradling, 2004; Lucchetta and Ohlstein, 2017). For example, in the mammalian intestine, diverse cell types of the secretory and absorptive lineages can undergo reversion to stemness in response to assaults, including irradiation, chemotherapy, helminth infection, and Dextran Sodium Sulfate (DSS)-induced colitis (reviewed in de Sousa e Melo and de Sauvage, 2019). Intriguingly, this response includes re-activation of a number of genes associated with fetal development (Nusse et al., 2018; Yui et al., 2018), a phenomenon also observed in the stomach (Fernandez Vallone et al., 2016) and corticospinal tract motor neurons (Poplawski et al., 2020). The capacity to restore the stem cell pool from non-stem cells is observed in tumors as well, but it exhibits striking context dependence. In particular, adenomas in the intestine arise from LGR5-positive stem cells (Schepers et al., 2012) but can be maintained by non-stem (LGR5-negative) cells when the stem cell pool is compromised (de Sousa e Melo et al., 2017). In contrast, LGR5-positive stem cells are critical for maintenance of intestinal-derived liver metastases (de Sousa e Melo et al., 2017; Fumagalli et al., 2020; Figures 4E and 4F).

Although many of the cell types that have been shown so far to revert to stemness are not terminally differentiated, recent work has revealed that cells exhibiting highly specialized functions that, under homeostasis, contribute at very low levels to tissue repopulation are able to re-enter the cell cycle and repopulate the tissue following damage. In the intestine, post-mitotic Paneth cells can re-enter the cell cycle and produce progeny in response to inflammation (Schmitt et al., 2018). Similarly, Chief cells in the stomach and hepatocytes and cholangiocytes in the liver can drive dramatic expansion of tissue in response to damage (Font-Burgada et al., 2015; Leushacke et al., 2017; Stange et al., 2013). Finally, compensation for lost functions is not restricted to recovery of stem cell functions because, when Paneth cells are ablated in the intestine, enteroendocrine and tuft cells can adopt the Paneth cell position and provide some of this cell type's stem-cell-supporting functions (van Es et al., 2019). Together, these studies have demonstrated that many cell types have significant potential to adopt features and functions beyond those observed in homeostasis, which can be unleashed in response to experimental perturbations and damage.

Modulation of Cellular Contributions through Competitive Interactions with Neighboring Cells

An additional example of context-dependent cell function is cell competition, in which the identity of neighboring cells determines whether a cell expands in the tissue or is eliminated. In particular, cells that contribute to tissue function when surrounded by genetically identical cells may be actively eliminated when brought into contact with cells of increased fitness (Figures 4J and 4K). As a result, fitter cells become "winners" and expand and colonize the tissue at the expense of weaker cells, called "losers," which are eliminated through engulfment, apoptosis, extrusion, delamination, and differentiation (Figure 4K). Thus, the contribution of a cell to a tissue can be dramatically modulated by its fitness relative to its neighbors.

Cell competition was first described in the *Drosophila* wing imaginal disc (Morata and Ripoll, 1975). Flies heterozygous for mutations in ribosomal genes (termed *minutes*) are viable and fertile but have slower growth rates and minor structural abnormalities. Cells heterozygous for *minute* survive as long as the entire tissue is composed of heterozygous mutant cells. However, in the presence of wild-type cells, mutant cells are eliminated by apoptosis (Morata and Ripoll, 1975). Similarly, mice heterozygous for a ribosomal mutation (*bst*) are viable with minor defects, but *bst* heterozygous cells are eliminated when combined with wild-type cells in chimeras (Oliver et al., 2004). Thus, mutations that are seemingly inert when introduced throughout the tissue can confer a selective disadvantage when combined with cells of a distinct genetic complement.

A wide variety of mutations can enable a cell of a given genetic background to make substantially different contributions to the tissue, depending on its fitness relative to its neighbors. Many of these were first identified in *Drosophila*, as summarized in Figure 5 and reviewed in detail elsewhere (Baker, 2017; Bowling et al., 2019; Johnston, 2014; Nagata and Igaki, 2018). Subsequent work has identified cell competition in a variety of mammalian systems, including the hematopoietic system (Bondar and Medzhitov, 2010), epiblast (Clavería et al., 2013; Díaz-Díaz et al., 2017; Sancho et al., 2013), and embryonic skin (Ellis et al., 2019). Many studies have focused on cells harboring heterozygous loss-of-function alleles, which are eliminated when apposed to wild-type neighbors, as in the case of the key growth regulator *myc* and its related isoforms (Clavería et al., 2013; Ellis et al., 2019). Conversely, gain-of-function mutations, such as those that upregulate *myc*, can increase a cell's fitness relative to its neighbors, generating "supercompetitors" that expand at the expense of wild-type neighbors (Clavería et al., 2013; Moreno and Basler, 2004). Similar interactions may also be at play among stem cell populations. For example, in the mouse and human intestine, stem cells harboring oncogenic mutations preferentially replace their wild-type neighbors (Nicholson et al., 2018; Snippert et al., 2014; Vermeulen et al., 2013). However, in tissues such as the hair follicle, cell competition can also function as a tumor-suppressive mechanism, with wild-type cells suppressing

	Genotype	Win or Lose (compared to wt cells)	References
Hyperplasia or oncogenesis	*yorkie*(high dose, in testis cyst stem cells)	W	(Amoyel et al., 2014) (Huang et al., 2005)
	ras^{v12}(with *scrib*$^{-/-}$ or *lgl*$^{-/-}$ or *dlg*$^{-/-}$)	W	(Pagliarini and Xu, 2003)
	ptch$^{-/-}$ (in testis cyst stem cells)	W	(Amoyel et al., 2014)
	ex$^{+/+}$, *ft*$^{+/+}$, *sav*$^{+/+}$, *hpo*$^{+/+}$, or *wts*$^{+/+}$	W	(Tyler et al., 2007)
	apc$^{-/-}$ or *axin*$^{-/-}$	W	(Vincent et al., 2011) (Suijkerbuijk et al., 2016)
	egfr(high dose)	W	(Eichenlaub et al., 2016)
	dco^{3}	W	(Jursnich et al., 1990)
	ago$^{-/-}$	W	(Moberg et al., 2001)
	avl(low dose) and *crb*(high dose)	W	(Hafezi et al., 2012) (Lu and Bilder, 2005)
	bam$^{\Delta 86}$, *bgcn*^{20093} or *bgcn*^{20915} (in female germline stem cells)	W	(Jin et al., 2008)
Organ size maintained	*minute*$^{+/-}$	L	(Kucinski et al., 2017; Morata and Ripoll, 1975)
	yorkie$^{-/-}$	L	(Huang et al., 2005)
	scrib$^{-/-}$	L	(Brumby and Richardson, 2003)
	mahj$^{-/-}$	L	(Kucinski et al., 2017) (Tamori et al., 2010)
	lgl$^{-/-}$	L	(Tamori et al., 2010) (Menéndez et al., 2010)
	ept^{2}	L	(Moberg et al., 2005)
	tsc1(high dose) and *tsc2*(high dose)	L	(Potter et al., 2001)
	nrf2(high dose)	L	(Kucinski et al., 2017)
	brk(high dose)	L	(Moreno et al., 2002)
	dlg $^{-/-}$	L	(Igaki et al., 2009)
	azot$^{+/+}$	L	(Merino et al., 2015)
	vps25^{PB2931}	L	(Thompson et al., 2005)
	rab5(low dose)	L	(Ballesteros-Arias et al., 2014)
	fwe(low dose)	L	(Rhiner et al., 2010)
	csk$^{-/-}$	L	(Vidal et al., 2006)
	fz$^{-/-}$ and *fz2*$^{-/-}$	L	(Vincent et al., 2011)
	myc(low dose)/ *myc*(high dose)	L/W	(de la Cova et al., 2004) (Moreno and Basler, 2004)
	stat(low dose)/ *stat*(high dose)	L/W	(Rodrigues et al., 2012)
	lgl$^{-/-}$ or *dlg* $^{-/-}$ (in ovary follicle stem cells)	W	(Kronen et al., 2014)
	socs36E(high dose, in male germline stem cells)	W	(Issigonis et al., 2009)

Figure 5. Overview of Cell Competition Mutants in *Drosophila*
Studies of cell competition in mammals have taken advantage of the extensive groundwork laid by research in flies. The table summarizes mutations that have been identified in fly to confer winner (W) or loser (L) status when combined with wild-type (wt) cells. Mutations in which expansion of the winner cells drives organ enlargement are indicated in purple; mutations that result in competition but maintain organ size are indicated in blue.

the outgrowth of clones harboring pre-oncogenic mutations (Brown et al., 2017b; Pineda et al., 2019). Broadly, these studies have revealed a wide variety of tissues and mutational insults in which cellular contribution depends on the relative fitness of other cells within the tissue.

Additional studies have added a further layer of complexity to a cell's functional contribution relative to its neighbors by demonstrating that the same mutations can impose both winner and loser status, depending on the physiological state of the organism. For example, during development, cells with high activity of the transcriptional regulator yes-associated protein (YAP) are winners, whereas adult cells overexpressing YAP are losers (Chiba et al., 2016; Hashimoto and Sasaki, 2019). Additionally, environmental factors, such as nutritional intake, can also reassign winner and loser status. For example, several epithelia have been shown to eliminate cells harboring *ras*^{v12} mutations when combined with wild-type cells (Kon et al., 2017). However, when mice are fed a high-fat diet, elimination of *ras*^{v12} cells from the intestine and pancreas is suppressed, resulting in tumor-like masses (Sasaki et al., 2018). Thus, a cell's functional contribution varies according to its fitness relative to its neighbors, but this, in turn, varies in diverse physiological contexts.

Together, these studies reveal that cell identity is highly sensitive to the context in which it is assessed. In addition to the genetic, disease, and damage contexts highlighted here, extensive work is examining additional factors that modulate cellular features and functions, such as age (reviewed in Keyes and Fuchs, 2018; Figures 4G–4I), nutrient availability and/or utilization (reviewed in Intlekofer and Finley, 2019; Mihaylova et al., 2014), microbes (reviewed in Larsen et al., 2020), sex (Hudry et al., 2016, 2019), and beyond (Figure 4L). Moreover, it is emerging that past experiences, such as inflammation, can be stored as epigenetic memories that influence future cellular functions (Naik et al., 2017). These studies underscore key limitations to extrapolating cellular functions across conditions and open up a wealth of new biology regarding how cells coordinate and compete to maintain and regenerate organs over the lifespan.

Conclusions and Outlook

The rapid advances of diverse technologies are dramatically expanding the dimensions along which cell identity can be defined. These studies reveal the cellular composition of tissues with increasing resolution and also point toward strategies to coax cells to adopt features and functions of therapeutic value. As the number of molecular fingerprints assigned to cell types grows, so too does the number of different contexts in which cell function is being assessed across diverse injury and disease models and genetic combinations. Together, our rapidly expanding capability to detect features and functions are revealing that "cell types" that were perceived as monolithic and stable in fact represent composites of multiple cells with distinguishable molecular signatures and have the capability to adopt new features and functions in new contexts.

From the perspective of identifying cells based on molecular features, the numerous potential modalities for cell profiling present opportunities and challenges. These approaches are generating more complete perspectives on cellular designations and provide fundamental insights into the causal mechanisms that drive phenotypes. Major challenges for ongoing work are to integrate the molecular profiles defined through these numerous different technologies and to understand why different modalities may not converge to identify the same delineations between cells. Finally, a key goal is to understand how subdivisions of molecular fingerprints between cells translate to functional consequences.

Functional assessments of cell types in diverse contexts are continuing to reveal that cells have the capability to adopt features and functions beyond those observed in homeostasis, and these can be uncovered by experimental and pathological perturbations. These observations suggest that, in contrast to a model connecting one cell type to one function, cell types might instead be more accurately described as a suite of potential functions that can be unleashed in specific contexts. From this perspective, and given the role that plasticity can play in disease progression, an intriguing question is what sets the boundaries on those functions. How is plasticity constrained in healthy tissue? How is plasticity reverted after damage is resolved? Experiments demonstrating how microenvironmental changes can shift cellular features and functions continue to raise important questions regarding the extent to which cell function is hardwired, calling to mind Sydney Brenner's evocative description of cell specification based on ancestry (the so-called European plan) or based on neighbors (which he called the American plan) (referenced in Fraser and Harland, 2000). In this regard, the wealth of techniques described here that preserve spatial context while performing high-dimensional profiling lay exceptional groundwork to dissect the interplay between intrinsic and extrinsic features driving cellular functions.

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AUTHOR CONTRIBUTIONS

K.L.M. and D.C.-A. conceptualized, researched, and wrote the article with input from O.D.K.

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