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MECHANISMS OF CELL DIVERSIFICATION

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Mechanisms of Cell Diversification

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Cell identity and function is determined by the intrinsic wiring of the gene regulatory network that endows progenitors with the competence to respond appropriately to extrinsic cues in a spatiotemporally-dependent manner. One such class of cues, morphogens, instruct cells in their identity by virtue of a concentration gradient, but how this is interpreted at gene regulatory levels to result in sharp and robust boundaries of gene expression is poorly understood. The patterning of the dorsoventral (DV) axis of the developing vertebrate nervous system by Sonic hedgehog (Shh) and its bifunctional transcriptional mediator, Gli, results in the specification of distinct neural subtypes and serves as a model of morphogen function.

The identification and functional analysis is described of *cis*-regulatory modules (CRMs) required for the neural-specific interpretation of morphogen activity by genes that pattern the dorsoventral axis of the CNS and coordinately specify progenitor subtype identity. The results presented are consistent with a model in which morphogen exposure is interpreted via distinct transcriptional mechanisms by genes induced close to the morphogen source as compared to those induced at long-range. In particular, long-range genes directly interpret the Gli repressor (GliR) gradient, resulting in target gene derepression in response to Shh. As a result, expression of long-range targets is critically reliant on additional activators that act in synergy with Gli activators (GliA) as well as direct repressive input from other TFs that restrict expression to the ventral neural tube. By contrast, locally induced Shh target genes directly interpret the balance between GliA and GliR and require input by GliA for their expression. Although synergy with other activators is required for expression, locally induced genes appear to be largely insensitive to mutations of their Gli-binding sites. Evidence is provided that input from other morphogens that pattern the DV axis as well as from Hox proteins that regulate cell identity along the anteroposterior axis is directly integrated into the same set of Shh-regulated CRMs to modulate the relative sizes of progenitor domains along these axes.

The high dependence of local targets on the balance of Gli isoforms to regulate their range of expression obviates the need for other direct repressive input, and, consistent with this, genetic and gain-of-function evidence is presented that Pax6 cell-autonomously suppresses expression of local responses by upregulating *Gli3* and, hence, GliR. Conversely, the locally induced Shh target, Nkx2.2, is shown to cell-autonomously amplify the Shh response by downregulating *Gli3*. Extracanonical feedback modulation by Shh-regulated genes offers a mechanism for the phenomenon of cellular memory that is essential to produce qualitative responses to quantitative input, including previous observations that the highest Shh responses are not immediately accessible, but rather depend on ongoing morphogen exposure. Accordingly, whereas Pax6 suppresses floor plate (FP) differentiation, ectopic expression of Nkx2 proteins at early stages promotes FP differentiation in a Shh-dependent manner, whereas misexpression at later stages specifies p3 identity, and it is suggested that the loss of this ability reflects a temporal switch of progenitor competence.

Shh signaling is transduced through the primary cilium, which is absolutely required for stabilization of GliA and facilitates GliR formation. The differential sensitivity of local and long-range target genes to perturbed Shh signaling is consistent with the phenotypes of mutants that impact cilia morphology but do not prevent ciliogenesis. Mutants of *Rfx4*, which regulates ciliogenesis, display a selective reduction of the size of locally regulated domains. Surprisingly, this is due not to a delayed induction of local target genes, but rather to a failure to maintain them as Shh signaling declines. This period is characterized by reactivation and extended co-expression of Olig2 and Pax6 in Nkx2.2-expressing progenitors that do not commit to FP fate. It is suggested that this mixed identity corresponds to a metastable cell state that is acutely sensitive to ongoing fluctuations in morphogen exposure and required to generate sharp domain boundaries. Consistent with impaired Shh signaling, Rfx4 mutants fail to extinguish *Gli1* expression at the ventral midline, which is correlated with an extension to the ventral midline of the zone of Olig2/Pax6 reactivation and delayed FP commitment.

Evidence is presented that the neural-specific response of morphogen target genes is regulated by Soxb1 proteins, which are sufficient to induce these genes in the developing limb in response to Shh, retinoid, or Bmp morphogen exposure. Moreover, the collocation of Soxb1- and Gli-binding sites constitutes a genomic signature that reliably predicts the neural-specific expression of nearby genes.

LIST OF PUBLICATIONS

I. Oosterveen, T., Kurdija, S., Alekseenko, Z., **Uhde, C.W.**, Bergsland, M., Sandberg, M., Andersson, E., Dias, J.M., Muhr, J., and Ericson, J. (2012). Mechanistic Differences in the Transcriptional Interpretation of Local and Long-range Shh Morphogen Signaling. Developmental Cell *23*, 1006-1019.

II. Lek, M., Dias, J.M., Marklund, U., **Uhde, C.W.**, Kurdija, S., Lei, Q., Sussel, L., Rubenstein, J.L., Matise, M.P., Arnold, H.H.*, et al.* (2010). A Homeodomain Feedback Circuit Underlies Step-function Interpretation of a Shh Morphogen Gradient during Ventral Neural Patterning. Development *137*, 4051-4060.

III. **Uhde, C.W.**, Dias, J.M., Andersson, E., Jeggari, A., Kozhevnikova, M., Karlen, M., Peterson, A.S., and Ericson, J. Rfx4 Regulates the Local Interpretation of Shh Signaling in the Developing Nervous System. Manuscript.

IV. Oosterveen, T., Kurdija, S., Ensterö, M., **Uhde, C.W.**, Bergsland, M., Sandberg, M., Sandberg, R., Muhr, J., and Ericson, J. (2013). SoxB1-Driven Transcriptional Network Underlies Neural-Specific Interpretation of Morphogen Signals. Proceedings of the National Academy of Sciences of the United States of America *110*, 7330-7335.

Additional publications not included in the thesis:

i. **Uhde, C.W.**, Vives, J., Jaeger, I., and Li, M. (2010). Rmst is a novel marker for the mouse ventral mesencephalic floor plate and the anterior dorsal midline cells. PloS One 5, e8641.

ii. Panman, L., Andersson, E., Alekseenko, Z., Hedlund, E., Kee, N., Mong, J., **Uhde, C.W.**, Deng, Q., Sandberg, R., Stanton, L.W., Ericson, J., Perlmann, T. (2011). Transcription factor-induced lineage selection of stem-cell-derived neural progenitor cells. Cell Stem Cell 8, 663-675.

iii. **Uhde, C.W.**, Ericson J. (*in press*). Transcriptional Interpretation of Shh Signaling: Computational Modelling Validates Empirically Established Models. Development.

CONTENTS

GLOSSARY OF TERMS

allele	one of the two parental copies of a gene
apoptosis	programmed cell death
bistability	See *multistability*; $n = 2$
blastula	preimplantation embryo from which ES cells are derived
chimaera	an embryo or adult composed of cells that originated from two different organisms, which can be from different species following manipulation
cilium	cellular organelle that can, in principle, mediate a variety of functions, e.g. motility, signal transduction
clone	a population of cells originating from a single founder cell
dorsal	Back (i.e. top) side of an organism or tissue
ectoderm	presumptive skin and nervous system
electroporation	an experimental manipulation in which synthetic DNA molecules are taken up by cells upon application of an electric field
equivalence	the ability of a cell to assume the role of another
expression	the transcriptional status of a gene
haploinsufficient	gene dosage effect in which both alleles are required for normal gene function
homology	DNA sequence similarity; *orthology*: degree of ~ of a given gene across species; *paralogy*: degree of ~ between related genes within a species
hypomorph	a mutant in which the function of a gene is not completely abolished
lineage tracing	the marking of cells, e.g. with a dye or transgenically, in order to follow the location and identity of their progeny
marker	a gene whose expression is associated with a specific cell lineage, cell type, and/or function
metastable	(in the context of a GRN) a state that is stable enough to permit self-renewal but only for a limited time.
metazoan	multicellular animals
mitosis	cell division
morula	early embryo in which cells are ostensibly equivalent and that gives rise to a blastula
multistability	a property of a system whereby n distinct states are stable under the same conditions
reporter	a transgene used as an experimental readout of gene or protein activity, e.g. eGFP

topographical	(of a lineage) the birth of differentiated progeny at or near the location at which they are to function, such that little or no migration is required to reach it. May require generation of functionally similar cells at distant positions of the embryo.
transit-amplifying	progenitors that are committed, under normal circumstances, to a lineage but may still undergo a number of cell divisions.
transcriptome	the expression status of all genes in the genome within a cell or population
transposition	insertion of DNA into the genome. Typically by a virus.
typological	(of a lineage) the birth of differentiated progeny at or near the site of other functionally and ontogenetically related progeny. May require migration/transport to the site of function in the mature organism.
ventral	belly side of an organism or tissue

LIST OF ABBREVIATIONS

AP	Anterior-posterior
Bmp	Bone morphogenetic protein (Tgfß superfamily member)
bp	DNA Base pairs
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CRM	*Cis*-regulatory module
DBD	DNA-binding domain of a TF
DV	Dorsal-ventral
eGFP	Enhanced green fluorescent protein
ES cells	Embryonic stem cells
FP	Floor plate
GBS	Gli-binding site
GliFL	Full-length Gli
GliA	Gli activator
GliR	Gli repressor
GPCR	G protein-coupled receptor
GRN	Gene regulatory network
HB	hindbrain
HBS	HD-binding site
HD	Homeodomain class DBD
Hh	Hedgehog
ICM	Inner cell mass (of the preimplantation embryo)
kb	Kilobase pairs (1,000 bp)
Mb	Megabase pairs (1,000,000 bp)
MN	Motor neuron
PKA	Protein kinase A
pMN	MN progenitor
p3	V3 or vMN progenitor
r	rhombomere
RA	Retinoic acid
RAR	RA receptor
RARE	RA response element (RAR recognition site)
SBS	Soxb1-binding site
Shh	Sonic hedgehog
sMN	Somatic MN
TBS	Tcf-binding site
TF	Transcription factor
TSS	Transcription start site
vMN	Visceral MN

INTRODUCTION

CELLULAR DIVERSITY

All cells resemble one another; each individual cell is individual in its own way. An oft-quoted statistic is that there are roughly two hundred cell types in the human body[1], but according to this system of classification, all neurons are defined as a single cell type. Nevertheless, a casual perusal of the drawings of Santiago Ramón y Cajal, the father of neuroscience, reveals that neurons can vary enormously in appearance (figure 1). Consider sensory neurons that transmit environmental stimuli to the central nervous system and Purkinje neurons that coordinate movement: whereas the former are pseudounipolar, with relatively few connections to other neurons, the latter are multipolar and extraordinarily elaborate, each forming ~150,000 synapses with other neurons (figure 1A,B). Not only do such neuronal subsets look different; they are found at stereotypic positions and innervate target cells in a highly specific fashion, enabling distinct subsets of neurons to control functions as diverse as voluntary muscle movement, physiological homeostasis, the assembly of actions or words required for large multicellular organisms to communicate, and the deductive reasoning that led Descartes to declare, "Cogito ergo sum." As Mark Twain might have summarized the point, "There are three kinds of lies: lies, damn lies, and statistics."

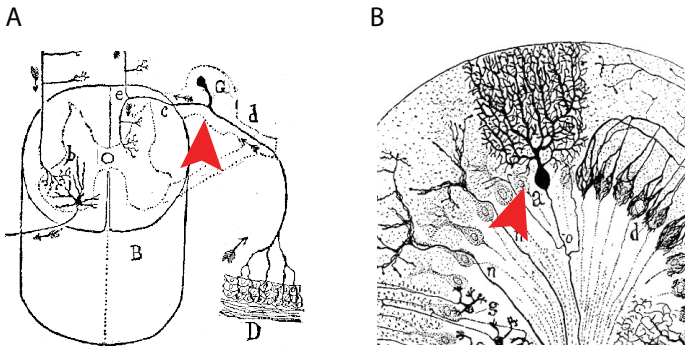

Figure 1. Illustrations by Ramòn y Cajal depicting types of neurons found in cross-sections of (A) the spinal cord, in which a sensory neuron is indicated (arrowhead) and (B) a portion of the cerebellum, in which a Purkinje neuron is indicated (arrowhead). Modified from Ramòn y Cajal, 1892 and 1894, respectively.

This might lead one to query just how far down the rabbit hole goes. Ultimately, morphological and functional differences between cells (much like people) are the result of their perception of environmental cues and consequent responses, which transpire according to constraints imposed by the genome. Genome-wide analyses of gene expression in defined cell types have therefore garnered much interest among biologists. Such an approach has been taken previously, for example, to fish for candidate genes expressed in motor neurons of the vertebrate hindbrain (Panman et al., 2011), indicating that, although some are expressed in all hindbrain motor neurons, many are expressed only in specific motor neuron subpopulations (figure 2). However, these data understate the complexity, as they merely report detectable expression as a binary (on/off) state, whereas gene expression varies qualitatively even between ostensibly equivalent cells (Novick and Weiner, 1957) and indeed within the same cell between gene alleles (Deng et al., 2014). The question of what differences constitute functional variation versus mere "noise" has only begun to be addressed (Altschuler and Wu, 2010),

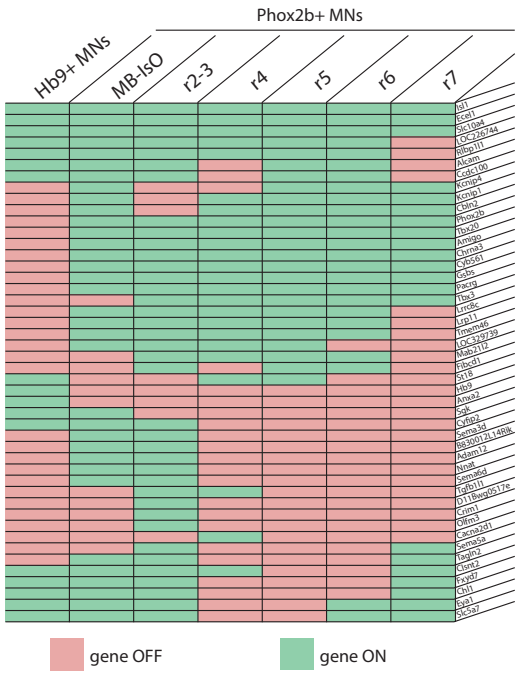

Figure 2. Unique molecular signature of motor neuron subpopulations visualized in a binary map of candidate gene expression within motor neuron subpopulations at e11.5, as determined by *in situ* hybridization. Genes ordered by manual hierarchical clustering according to co-expression with the each other in motor neurons.

but their potential to affect the behavior of a cell is proportionate to their amplitude, their coincidence with variation in the expression of other genes, and the functions of that collection of genes. Adding isotopic and quantum differences into consideration, one stands on firm enough ground to claim that no two cells have ever been, nor ever will be, exactly the same (although I hope the reader will allow that to prove it is beyond the scope of this thesis). That said, cells clearly have shared traits, many of which are exclusive to specific subsets, and classification is an essential tool for man to understand nature. Happily, one is not limited to a molecular descriptive in order to arrive at a satisfactory conclusion, which one might find with the following definition:

> A distinct cell type is one whose function in its own environment cannot be substituted by a cell of another type, with the important exception of a cell type that can perform the function of two or more otherwise distinct cell types[2], therefore itself constituting a distinct cell type.

The reader will note that this definition renders the task of defining the precise number of distinct cell types unknowable for the foreseeable future[3]; clearly, however, it is orders of magnitude greater than two hundred, and given the massive diversity of form and function of cells in the nervous system, it seems likely that a disproportionately large fraction will be found there.

NEURAL DIVERSITY AND FUNCTION

Understanding the logic that underlies how the plethora of cells found in the central nervous system (CNS) is organized and interacts to constitute such a powerful information processing system continues to present a grand challenge. Inevitably, most efforts to date have been limited to the study of its units, which, in the broadest terms, are classified as either neurons or glia.

Neurons are the core units of the nervous system (Kandel, 2013), and characterized by their unique and highly diverse morphologies (figure 3), which consist of (1) the soma, or neuronal cell body; (2) dendritic branches that extend from the soma and receive input from contacting cells; (3) the axon(s), or nerve fiber, which extends from the soma to the innervation targets of the neuron, and can be over a meter long in humans; and (4) the presynaptic terminal, which, together with the postsynaptic terminal of a neighboring neuron, forms a synapse: an interface between two neurons that enables the former to signal to the latter (Kandel, 2013). The appropriate stimuli initiate action potentials, i.e. electrical impulses that are propagated along the cell membrane of the neuron from the dendrites to the axon, in a Mexican wave of electric activity, ending at the axon terminals. At this

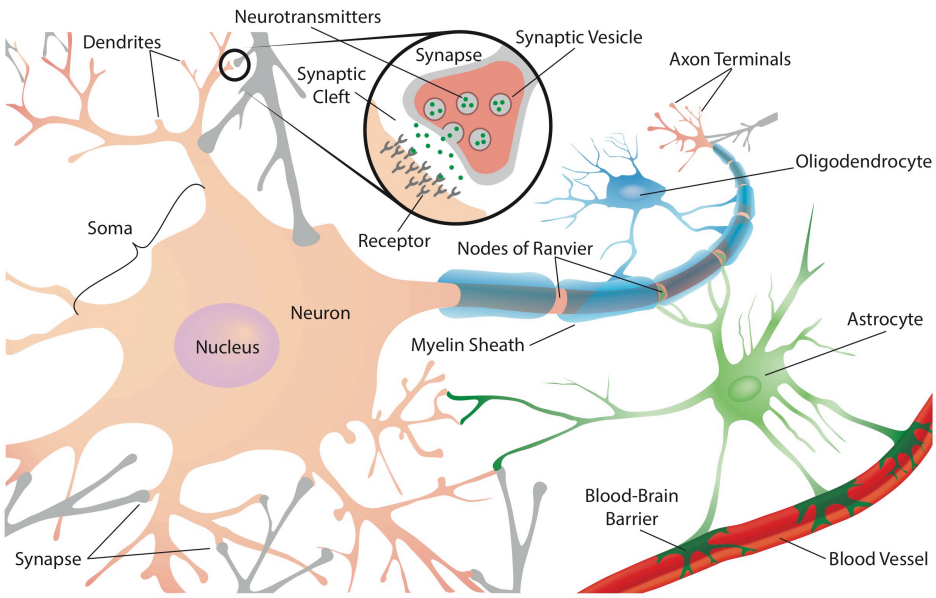

Figure 3. Schematic illustration of the most abundant adult neural cell types in the CNS. Neurons are electrically excitable cells with specialized morphological features that include dendrites emanating from the soma, and at least one axon with terminals on other neurons or muscle fibers. Connections for communication between neurons are called synapses. Upon receipt of an action potential from the axon, synaptic vesicles at the presynaptic terminal move toward the synaptic cleft and release their neurotransmitter cargoes into this space, which then diffuse toward the postsynaptic terminal where they bind receptors, triggering or inhibiting an action potential in that cell, depending on the nature of the transmitter. Oligodendrocytes are found near the axons of many neurons, providing those cells with myelin sheaths, which insulate the axon to allow faster, saltatory propagation of action potentials. Astrocytes regulate neurotransmission at the nodes of Ranvier and synaptic transmission, as well as forming the blood-brain barrier, among other functions. Adapted from original, available on Wikimedia, by Ruiz-Villarreal.

point, typically, the arrival of the action potential triggers the release of synaptic vesicles, which move to the presynaptic terminal and fuse with the plasma membrane, releasing their cargo of chemicals, called neurotransmitters, into the synapse. These travel across the synaptic cleft to receptors on the postsynaptic membrane of the neighboring neuron, triggering an action potential on that cell in the case of excitatory input, or preventing the initiation of an action potential in that neuron in the case of inhibitory input (Kandel, 2013). As one would intuit, neurons are evolutionarily the more ancient class of neural cell, present in all reported metazoan lineages except sponges and placozoans (Hartline, 2011), though even these organisms nevertheless possess most of the requisite genetic components (Sakarya et al., 2007), many of which are present even in unicellular organisms such as yeast and various bacteria, where they serve other, functionally related, purposes (Ryan and Grant, 2009; Verkhratsky and Butt, 2013).

The evolution of increasing neuronal specialization necessitated an increasing reliance on the support of neighboring cells (Kettenmann et al., 2013), and glia have evolved in tandem with neurons to perform a wide array of functions in service to them (figure 3). By definition, glia comprise all non-neuronal cells located in the CNS, and include[4] (1) the morphologically similar but functionally distinct neuroepithelial progenitors and radial glia, discussed further below, with the latter also serving as a scaffold for migratory neurons; (2) oligodendrocytes, which increase the speed of neurotransmission by producing myelin sheaths that electrically insulate segments of axons by virtue of their lipid-rich composition, enabling saltatory neurotransmission; (3) ependymal cells, which produce and are required for the circulation of cerebrospinal fluid; and (4) astrocytes, which have a multitude of functions including (i) homeostasis of the nervous system through maintenance of the blood-brain barrier and distribution of nutrients from the blood; (ii) regulation of synaptic transmission; and (iii) regulation of neurotransmission at the nodes of Ranvier, between adjacent myelin sheaths (Verkhratsky and Butt, 2013).

Coincident with (or most likely incident to) the centralization of the nervous system in bilaterians (Verkhratsky and Butt, 2013), phylogenetic evidence indicates that glia are a uniquely bilaterian innovation, if not universally so, and, indeed, suggests that glia may have arisen independently in and/or been lost from various phyla, being, for example, present in our own Chordata and absent from the (relatively) closely related Hemichordates, yet present in the far more distantly related flatworms, Platyhelminthes (Hartline, 2011). The first vertebrate-like glia to appear during evolution are neuroepithelial progenitors, which can also be found in the sea urchin's phylum, Echinodermata (Verkhratsky and Butt, 2013). By far the most numerous and diverse adult glial cell class is the astrocyte, found in all vertebrates. Most phylogenetic analyses of astrocytes to date have been based on a highly limited set of marker genes, so it is difficult to be certain about the prevalence of these cells in bilaterians, but their appearance seems to be associated with and may be a prerequisite for increasing complexity of the nervous system. Indeed, astrocyte-like glia may have evolved on multiple occasions (Kettenmann et al., 2013). Ependymal cells are found throughout phylum Chordata, and this, together with the fact that their basal processes typically contact the remnants of embryonic blood vessels, has led to the suggestion that astrocytes could have originated by modification of an ependymal cell program (Kettenmann et al., 2013). Oligodendrocytes, meanwhile, are present in all jawed vertebrates but absent from jawless vertebrates such as lampreys, and their appearance has been implicated in the ability of the former to grow to large sizes, as they allow fast neurotransmission along thin axons, reducing the volume and weight of the CNS and peripheral nerves (Verkhratsky and Butt, 2013).

The developing vertebrate nervous system serves as a model for the study of mechanisms of cell fate determination, with especial focus on the neuronal diversity found in the spinal cord. These neuronal subtypes are comprised of (1) motor neurons (MNs), which extend their axons outside of the nervous system, innervating muscles to control their contraction and relaxation; (2) four classes of ventral interneuron (IN) subtypes, named V0-V3 INs; and (3) six classes of early born dorsal IN subtypes, named dI1-dI6, as well as an additional two classes of late-born dorsal INs, named dILa and dILb (table 1; Goulding, 2009). The designation of ventral and dorsal is based on the spatial positions of the developing spinal cord at which these various populations are born (figure 4).

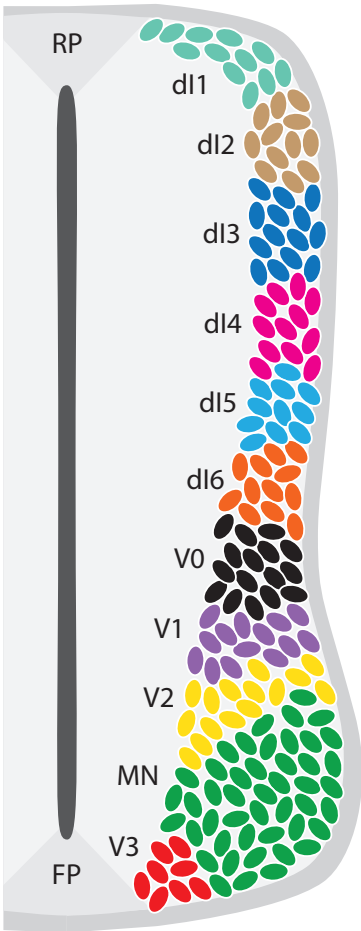

Figure 4. Schematic of the righthand side of the developing mouse spinal cord c. 10 days post coitum, depicting classes of early-born spinal neurons at their respective sites of birth on. The ventral side is at the bottom, dorsal the top.

The major roles of this collective group of cells are to centralize sensory information and either (a) respond reflexively and subsequently send it to the brain or (b) deliver it to the brain and subsequently execute directives therefrom, resulting in coordinated motor outputs, e.g. locomotion and respiration. Such outputs are regulated by neuronal networks termed central pattern generators (CPGs; figure 5; Kiehn, 2011). Of all the aforementioned cardinal classes of interneurons, all but two IN classes (dI1-2, which participate in ascending pathways to the brain) are thought to contribute to the CPG for locomotion, being either known components or forming synaptic contacts with those components (Alaynick et al., 2011; Vallstedt and Kullander, 2013). CPGs consist of two key neural outputs: rhythm and pattern (figure 5; Kiehn, 2011). The rhythm generator, or pacemaker, is required for rhythmic locomotion without repetitive orders from the motor cortex (which otherwise would probably feel rather tediously like starting to run at every step). It has recently been shown that ipsilaterally-projecting excitatory INs expressing Shox2 are required, at least in part, for rhythmic locomotion (Dougherty et al., 2013) The patterning of locomotion determines the alternation of appendages, flexion versus extension of jointed limbs, or alternation of muscles of the body wall in swimming and slithering animals. The CPG left-right alternating system has evolved extensively among vertebrates, with some fish, such as lampreys, possessing a relatively simple, continuous CPG throughout the spinal cord for coordinated axial movement in swimming, whereas tetrapods have a segmented CPG that powers locomotion via the forelimbs and hindlimbs, while axial regions control respiratory movements of the thorax and abdomen by virtue of a modified swimming CPG (Goulding, 2009). Pattern is chiefly coordinated by commissural INs, i.e. neurons that project axons to the opposite half of the spinal cord and either inhibit or excite the neuronal network for the contralateral muscle, leading to alternating or synchronous locomotion of paired appendages, respectively (figure 5). Moreover, commissural INs with ascending projections have been implicated in the coordination of hindlimbs with forelimbs (Kiehn, 2006). Ipsilaterally-projecting inhibitory INs terminate each rhythmic phase, but the evolution of jointed limbs added an extra layer of

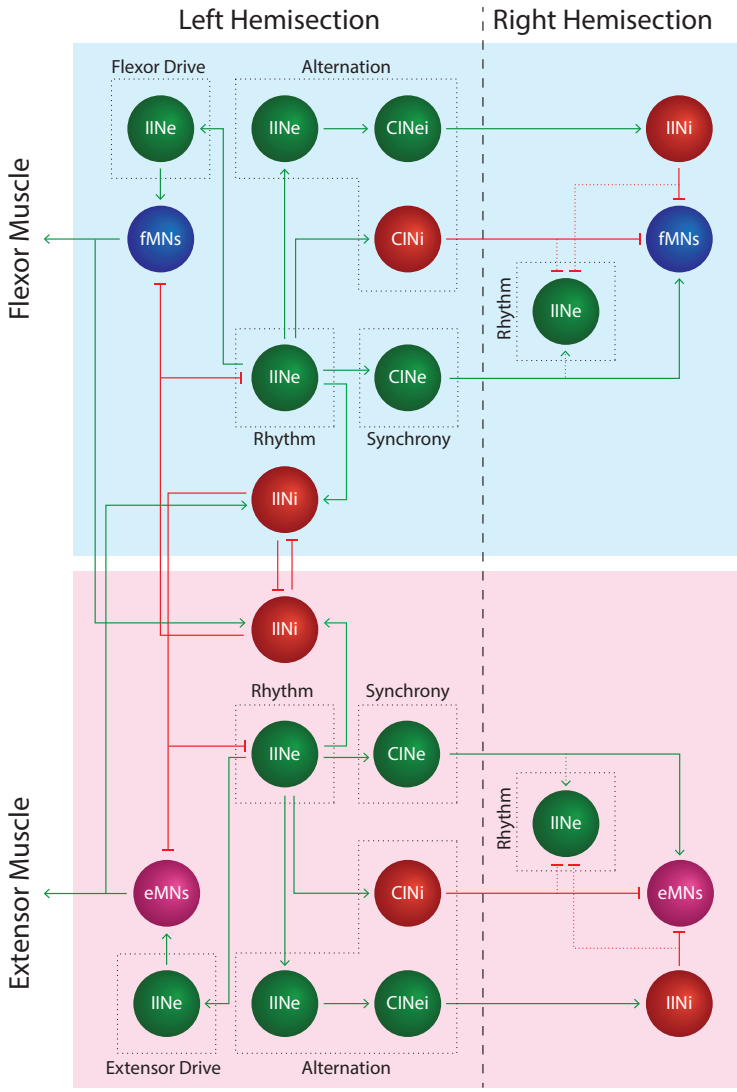

Figure 5. Schematic of the basic logic of the mammalian locomotion central pattern generator at limb levels of the spinal cord. The CPG is organized into de facto modules: left and right (demarcated by dashed line) as well as flexor (blue area) and extensor (pink area) modules. Excitatory IN populations and their output shown in green; inhibitory populations and output shown in red; MNs shown in blue/pink. Rhythm-generating cells set the frequency, or pace, of locomotion and the phasic nature of their activity is permissive in the selection between different modules. The rhythm kernel activates (1) IINe populations that provide excitatory drive to MNs (blue/pink); (2) either the CINe population, leading to synchronous left-right coordination, or the CINi population, suppressing activity of the contralateral MNs; (3) the IINi population in the same flexor/extensor module, suppressing the opposing module. In addition, at higher speeds, the IINe alternation population is also activated, leading indirectly to contralateral inhibition via CINei and contralateral IINi populations. Conceptually, both left and right modules and flexor and extensor modules are mirror images of each other. Ascending/descending inputs from other spinal cord levels and the brain, as well as the reflex arc are not shown.
fMNs, flexor MNs; **eMNs**, extensor MNs; **IINe**, ipsilateral excitatory INs; **IINi**, ipsilateral inhibitory INs; **CINi**, commissural inhibitory INs; **CINei**, indirectly inhibitory commissural excitatory INs

complexity, as flexor and extensor modules must also be selected in a mutually exclusive fashion, and here, too, these neurons inhibit activation of the opposing module to produce flexor-extensor alternation (figure 5; Kiehn, 2011). Robust commitment to muscle contraction requires motor neuron drive, which is provided by ipsilateral excitatory interneurons, which are themselves regulated by the rhythm kernel (Dougherty et al., 2013).

Neuronal classes in the more anteriorly situated hindbrain are broadly similar, the greatest difference occurring in the p3 domain, which, at most levels, initially generates visceral MNs that innervate visceral ganglia to control involuntary movements, e.g. dilation of the pupil and heart rate, or branchiomotor neurons that innervate the muscles of the face (Cordes, 2001). For the sake of brevity, these motor neuron subtypes will hereafter be abbreviated vMNs. In addition, while the pMN domain of the hindbrain generates somatic MNs (sMNs) as it does in the spinal cord, it is actually absent from the majority of anteroposterior hindbrain levels (Pattyn et al., 2003b). The midbrain is somewhat more divergent, perhaps the best known example being the generation of dopaminergic neurons from the ventral midline, which is occupied by the floor plate in the developing hindbrain and spinal cord, as well as in the midbrain at earlier stages (Andersson et al., 2006).

Recent efforts to dissect the neural circuitry underlying locomotion have been directed at the use of various mutants that enable the selective elimination, inhibition, or activation of specific neuronal cell types by taking advantage of molecular markers that distinguish between these cell types, many of which were identified in the context of developmental studies (Goulding, 2009). Thus, the locomotor CPG is illustrative of the fact that cellular diversity among even comparatively similar cells is critical to support essential functions of the organism, and underscores the utility of a thorough understanding of the development of cell identity in the functional analysis of physiological systems.

BIOGENESIS

To understand how cell diversity arises and is organized to create a fully developed organism is the fundamental challenge of Developmental Biology, but before microscopic observations would permit the problem itself to be properly formulated, it began to be addressed by the boundless imagination of the ancient Greeks in answer to that eternal query, "Daddy, where do babies come from?" The geometer Pythagoras (~570-495 B.C.) is credited as the first to address the anthropological question, a distinction of dubious merit, given that the attributed contribution was essentially to postulate that all the heritable characteristics of the offspring originated from the father, with the mother providing only a material substrate (Coward and Wells, 2013).

	Early Preformation	Epigenesis	Late Preformation	Scientific Consensus
Religiosity	atheistic	theistic	theistic	formal agnosticism
Predictability	deterministic	indeterministic	deterministic	indeterministic
Material	atomistic	divisibility ad infinitum	atomistic	atomistic
Biogenesis	preformed	epigenetic	preformed	hybrid

The philosophers of antiquity based their theories of biogenesis on the key observations that life is characterized by growth, and many traits are heritable, as well as certain assumptions about the nature of the Universe, which could conceivably have consisted, broadly speaking, of three categories of two mutually incompatible positions each: (1) religiosity, either theistic or atheistic; (2) predictability, deterministic or indeterministic; and (3) material, atomistic or divisibility *ad infinitum* (see Table 1). Democritus and Epicurus were among the earliest recorded philosophers to deliberate on the question in detail, as an adjunct to ancient atomic theory (Rieppel, 1986). The Atomists posited that indivisible matter, or atoms, were contributed by each parent from each part of the body and miniaturized[5], accounting for the multitude of possible traits inherited from each parent when combined in the offspring and resulting in a preformed organism that required only growth to reach its ultimate size (Rieppel, 1986). Heavily influenced by his mentor, Plato, Aristotle, on the other hand, came to the conclusion that Preformation was an insufficient and ultimately incorrect explanation of the generation of living creatures[6], pointing out, for example, that a baby boy is not a miniature man, beard and all, nor does he inherit any mutilations from any his parents may have had (Rieppel, 1986). Looking for a model of early development that could be readily studied in the 4th century B.C., Aristotle studied the chicken egg, and reported an undifferentiated mass that gradually acquired form following fertilization, one part after another, beginning with the heart (figure 6A; Maienschein, 2012; Rieppel, 1986). Borrowing from Pythagoras, he viewed the male contribution as the causative, or instructive, agent, but in contrast allowed the maternal contribution heritability (Maienschein, 2012). From these two schools the debate for the next two millennia was shaped, though it would involve some substantial deviations from their originators' ideas.

Both sides were to make great efforts to reconcile their views with Christian theology, and chief among these theists was William Harvey, who, in the mid 17th century, took up the study of the chicken egg again, as well as deer development[7], and concluded (incorrectly) that *ex ovo omnia* (Harvey, 1651). He elaborated on Aristotle's theory, substituting the generative force with God, and describing how the embryo developed by budding and compartmentalization, building structures on what had come before, and which he therefore named Epigenesis (Rieppel, 1986). While many of Harvey's assertions would prove to be correct, debate raged on, for Descartes had developed the phi-

losophy of Mechanism, in which matter was proposed to behave mechanically, much as a pendulum clock (Hatfield, 2014): in a nod to the deterministic ideas of the Atomists of antiquity, his disciples argued that the component parts of the organism were so functionally interconnected as to be impossible for any one to come into being independently of the others. This was fundamentally at odds with the epigenesists. Following the discovery of spermatozoa in 1677 (Gilbert, 2014), the Preformationists largely had the upper hand, as it became clear that the embryo did not develop from a disorganized, undifferentiated mass; indeed, its culmination was Hartsoeker's now much-maligned but iconic 1694 illustrations of homunculi (figure 6B; Maienschein, 2012), following conjectures of miniature individuals nested inside sperm cells like Russian dolls, *ad infinitum* (Correia, 1997). Improvements in the resolution of microscopes to reveal cell structure, together with the discoveries of evolution and genetics, ultimately ruled out strict Preformation and Epigenesis, development rather initiating at fertilization from the structured cell, predetermined[8] by the DNA 'blueprint', proceeding thereafter to complexity by the production and addition of further simple structures (Maienschein, 2012).

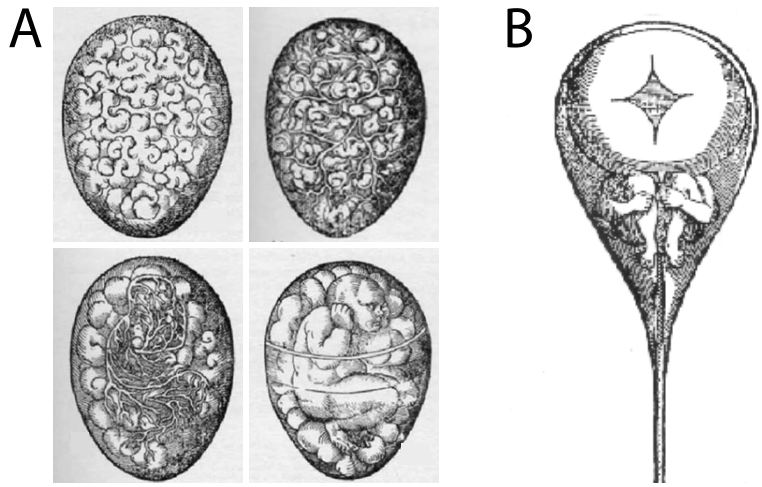

Figure 6. Epigenesis versus Preformation.
(A) Depiction of several stages of Aristotelean epigenesis by Jacob Rueff, as reproduced in A History of Embryology, (Needham, 1959). The newly fertilized human egg was assumed to resemble the chicken egg, and considered an undifferentiated mass (top left), from which the heart and vasculature were though to take shape (top right), with the other parts following thereafter (lower left), ultimately giving rise to the fetus (lower right).
(B) A homunculus, as depicted in *Essay de Dioptrique*, by Nicolas Hartsoeker, 1694.

MULTICELLULARITY AND THE ORIGINS OF STEM CELLS

"The only difference between the saint and the sinner is that every saint has a past and every sinner has a future." — Oscar Wilde

Cell Theory established that macroscopic organisms consist of collections of cooperating cells, but only a fraction of those cells give rise to subsequent generations, raising the question of how complex multicellularity from clonally developing single cells could have evolved from a unicellular ancestor, given that this entails the (typically vast) majority of cells in the group foregoing their chance to reproduce. Of course, the initial genetic variation of a dividing cell whose progeny fail to physically separate or detach is simple enough to explain, arising in our ancestors by as little as a single mutation of e.g. a cell adhesion molecule, increasing its strength of interaction (Bonner, 1998), or a failure to complete cytokinesis, leaving intercellular cytoplasmic bridges (Nedelcu, 2012). However, the emergence and stabilization of true multicellularity requires an understanding of the selective pressures and biological and physical constraints acting on the organism.

The essential functions of all living things contribute to one of two types of fitness: survival and reproduction (Nedelcu, 2012). Increases in individual size increase the scope for survival through a variety of advantages, e.g. the ability to avoid predation, or, indeed, to prey on others; however, the unicellular organism faces a number of size constraints, notably the need for efficient physiological exchange that depends on the surface area-to-volume ratio (Grosberg and Strathmann, 2007). By cooperating to form physical connections amongst each other, groups of cells circumvent this issue and, depending on what mutations occur in the future, subsequently enjoy substantial new possibilities for cooperation amongst group members. More immediately, however, a grouped structure creates economies of scale that further enhance survival[9] (Michod and Roze, 2001).

The purpose of life is genetic self-perpetuation (Dawkins, 1976), so it is to a cell's advantage to reproduce as often as possible, but the frequency of reproduction is limited by the need to survive long enough to do so, i.e. devoting time to foraging, evading predators, etc. (Michod and Roze, 2001). An illustrative example of such a trade-off (and they are numerous) that exists in most motile organisms, including all metazoans, is that between motility and mitosis: cells require a cilium for taxis and a mitotic spindle for chromosome segregation during cell division, both of which depend on the microtubule organizing center, of which there is only one per cell (Grosberg and Strathmann, 2007). Thus, a single-celled individual cannot purposefully move while dividing, but a group of cells may functionally segregate these functions at any given time into groups of motile and mitotic cells. In this situation, motile cells may be considered cooperative, contributing to group survival, whereas mitotic cells are behaving selfishly. Such a dynamic provides opportunities for cells to cheat, as mutations that shorten the cooperative phase of the cell cycle would result in an increased contribution of (cheating) progeny to the group relative to other cells (Michod and Roze, 2001). In the absence of the evolution of mechanisms to regulate such defections, subsequent generations of offspring would eventually not survive this cellular libertinism, as abundantly evident in the mortality rate of untreated cancer.

Fortunately for us, nature had an answer, and, rather typically, it was one that it had invented before and has used since. In contrast to mere colonial growth, multicellularity is defined as the stable integration of ancestrally solitary individuals "into a new functional, physiological, and reproductively autonomous and indivisible evolutionary unit - that is, a new kind of individual" (Nedelcu, 2012). Put another way, natural selection can act on both the lower level, in this case the cell, and the group level, here the colony or multicellular organism (Michod and Roze, 2001). Such multilevel selection is a hallmark of each of the seven major evolutionary transitions (Grosberg and Strathmann, 2007).

Recent experimental evolutionary studies have shown that clonal development evolves in the initial stages of the transition to clonal multicellularity (Hammerschmidt et al., 2014; Ratcliff et al., 2013), immediately providing opportunities for the evolution of altruistic behaviors through kin selection, which facilitates the toleration of mutations that increase group fitness at the expense of a given cell's fitness, provided that the benefit to group propagation and/or survival is high enough to withstand the loss of that cell and/or any of its potential progeny (Michod and Roze, 2001). It is worth emphasizing that altruistic behavior among cells is not voluntary; it is imposed, genetically. Thus,

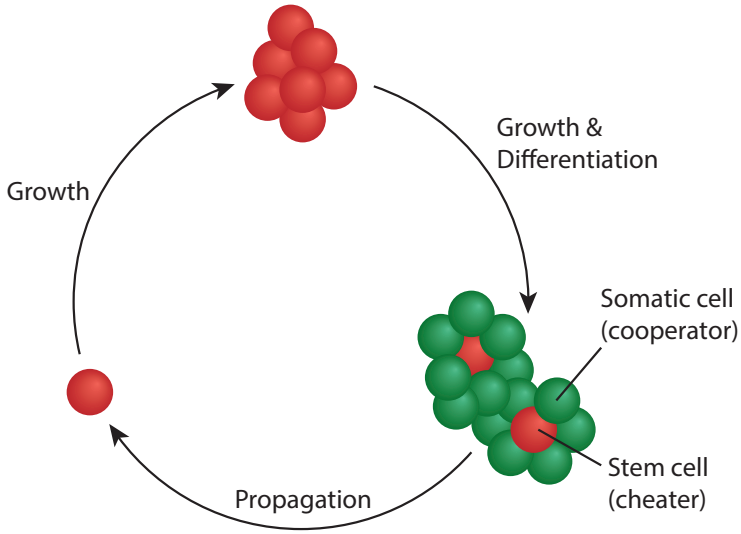

Figure 7. Life cycle of a multicellular organism with clonal reproduction.
By recursive growth and division, a single mitotic, non-motile cell gives rise to a colony or embryo comprised of mitotic cells. Most of these cells will differentiate into quiescent somatic cells that cooperate to promote group survival, which comes at the expense of their individual reproductive fitness. Some mitotic cells will remain as stem cells, however, and when embedded within the larger group of cooperator cells, these cells have been considered cheaters, as they avoid the personal costs of cooperation and thereby have the opportunity to reproduce (Michod & Roze, 2001). Recent studies indicate, however, that when reproduction is clonal, i.e. required to pass through a single-cell bottleneck, the need to evolve an efficient program of differentiation and propagation leads to the evolution of policing mechanisms that suppress true cheaters (Hammerschmidt et al., 2014).

mutations leading to altruism promote conflict resolution between higher and lower levels of selection in favor of the higher level, or 'greater good'. Such mutations include those that provide for the prevention, policing, or penalization of cheaters, which may be regulated by cell intrinsic or extrinsic mechanisms. For example, apoptosis is a form of intrinsically regulated punishment, whereas the suppression of cancer by the immune system is a means of extrinsic regulation and policing (Michod and Roze, 2001). Needless to say, these new functions do not materialize through a *de novo* evolutionary appearance of the necessary genetic components or pathways, but rather by baby steps: through the co-option of those already present to create novel functions (Nedelcu, 2012). Indeed, prerequisite traits for multicellularity, e.g. intercellular communication and apoptosis, are also present in unicellular organisms(Alberts, 2015), and, moreover, it has been shown that the altered regulation of only a small number genes is sufficient to control the selection between unicellularity and multicellularity in the various species of volvocacean algae (Kirk, 2003), betraying the ease with

which multicellularity can evolve[10]. That said, another prerequisite is the presence of sufficient genetic variation in the initial population, as the majority of these individuals will not be able to adapt to the conditions leading to multicellularity, and their lineages will consequently be extinguished (Darwin, 1859; Hammerschmidt et al., 2014).

The key innovation for the evolution of complexity, however, prevented cheating in the constituent population by sequestering the ability to proliferate within a subpopulation of cells, i.e. stem cells, through specialization, or cell differentiation. This gave rise to massive increases in the synergies that arise from economies of scale, and therefore made cheating more costly, as it is difficult to compensate for the function of specialized cells when they are disrupted (Nedelcu, 2012). At first blush, it appears paradoxical that a clonal mode of reproduction could successfully employ what are, in a sense, cheating cells to initiate development of a new organism, yet empirical studies suggest that is fundamentally required for selection to act on the higher, group level because it leads to a cell specialization (Hammerschmidt et al., 2014) and creates a bottleneck to prevent mutations to group fitness (Michod and Roze, 2001). The real challenge, then, would have been to evolve a means of transitioning between the initial mitotic cell type and the later predominantly quiescent cells of the mature organism (Hammerschmidt et al., 2014). Nevertheless, the discerning reader will have already noted that differentiation was, after a fashion, first a feature of unicellular organisms, with the generation of motile and mitotic cells occurring exclusively over time as opposed to space and time as characterizes multicellular organisms. Thus, a single mutation could have been sufficient to accomplish the necessary coupling of e.g. an intercellular communication pathway to the gene regulatory cascade promoting motility or preventing mitosis, creating the first terminally differentiated cell.

STEM CELLS

The explosive growth of the stem cell field has yielded an inconsistent usage of key terminology occasionally verging on the chaotic, so to avoid potential misunderstandings, a few definitions are in order. Stem cells are characterized by the ability (i) to continually self-renew, i.e. to produce daughter cells that are identical to the mother cell through cell division, and (ii) to differentiate into cells with distinct, more restricted properties (Smith, 2006). Although it is increasingly viewed as plausible that all cells capable of self-renewal and differentiation can be considered stem cells (Lander, 2011a), this must formally be proven on a case-by-case basis, until which time they are referred to as progenitors (Smith, 2006) and assumed to have the same general properties as *bona fide* stem cells. The terms 'stem cell' and 'progenitor' may therefore be used interchangeably hereafter, except in the context of specific cell types.

Key Terminology

potential	the range of fates into which a stem cell can ultimately differentiate
specification	a state in which a particular fate or set of fates is made available by environmental cues. These do not necessarily include the ultimate fate of the cell, as this may yet be determined by subsequent environmental events
competence	(i) the cell types into which a stem cell can differentiate at a given time; (ii) the ability of a cell to respond to specific signals that instruct in/select cell identities
determination	"specialized fate is fixed but the overt demonstration and realization of that fate has not yet become apparent" (Maclean & Hall, 1987)
commitment	the "relatively stable dedication to a specialized cell fate, either [determined] or realized" (Maclean & Hall, 1987)

Potential

Although the existence of stem cells was first proposed over a century ago (reviewed in Ramalho-Santos and Willenbring, 2007), an understanding of the mechanisms underlying their ability to differentiate has been slow to follow. A key issue is the nature of stem cell potential, i.e. the number of different cellular lineages to which a stem cell is ultimately capable of contributing. Without the benefit of hindsight, there are two basic ways in which stem cells could be imagined, from a bioengineering perspective, to generate the various cell types of the body: either a single stem cell type would have the potential to directly undergo terminal differentiate into all cell types, or stem cells would progressively differentiate along increasingly lineage-restricted branches (figure 8A). It will become increasingly clear in this discussion that although the latter alternative would be much less versatile, it requires far fewer components to robustly regulate its developmental program, as well as to be active within any given cell. From an evolutionary perspective, the former would therefore be far less likely to evolve in complex organisms.

Observations of embryonic development and cell transplantation experiments have largely determined the central principles of stem cell potential, initially establishing that (i) tissue and cell specialization is progressive[11], with a hierarchically branching lineage topology, perhaps nowhere better illustrated than in the Nobel Prize-winning lineage mapping of the 959 cells comprising the hermaphrodite roundworm, *Caenorhabditis elegans* (figure 8B; Sulston et al., 1983); (ii) not all stem cells of a tissue

have the same potential, exemplified by the variable degree to which haematopoietic stem and progenitor cells can reconstitute the different lineages of the blood following transplantation into lethally irradiated hosts (Abramson, 1977); and that (iii) over the course of development, stem cells ultimately undergo commitment to a given lineage with more restricted potential, as demonstrated by transplantation of labelled early and late migratory neural crest progenitors, the latter of which cannot generate the full complement of cell types produced by the former (Artinger and Bronner-Fraser, 1992).

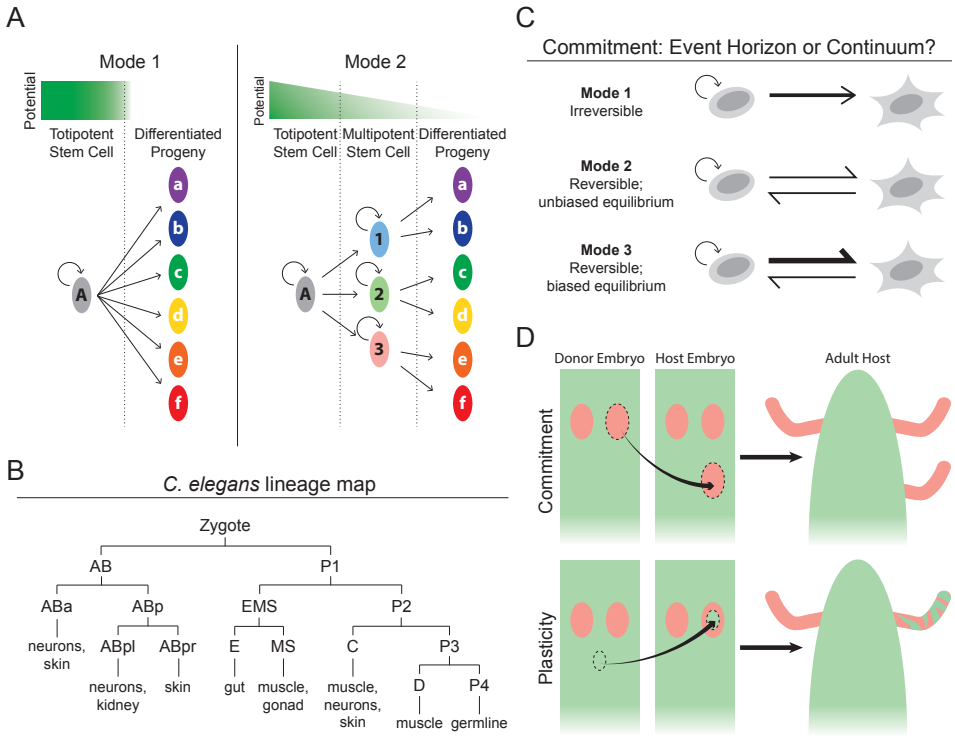

Figure 8. Potential and commitment.
(A) Alternative lineage topologies for generating differentiated progeny from a totipotent stem cell. Whereas in mode 1, the totipotent stem cell is competent both to self-renew and directly differentiate into any terminal fate, in mode 2, the totipotent stem cell can differentiate into multipotent stem cells with more restricted potential, capable of generating only a subset of terminal fates.
(B) The basal portion of the cellular lineage map of the nematode worm *C. elegans*, showing the first two to four cell divisions following fertilization. Note that a single, defined cell gives rise to the entire gut and germline after three and four divisions, respectively.
(C) Lineage commitment from stem cell to differentiated progeny could be envisioned as an irreversible event (mode 1), or a reversible event which can be transited forward or backwards with equal energetics (mode 2) or with an energetic profile favoring differentiation (mode 3). Adapted from Zipori, 2004.
(D) Schematic summarizing two key findings by Harrison (1918), in which grafting of one of the limb fields (pink) into an ectopic location gives rise to an ectopic limb, demonstrating that this tissue is committed to limb fate (upper panel), and in which grafting of other tissue (green) into the limb field leads to incorporation of the grafted tissue into the future limb, demonstrating the plasticity of the transplanted cells (lower panel).

Commitment and Plasticity

Differentiation could conceivably occur by (a) irreversible commitment, (b) via transitional states with an unbiased equilibrium, or (c) via transitional states with a biased equilibrium favoring differentiation (figure 8C; Zipori, 2004). The studies outlined above demonstrate that stem cells enjoy considerable autonomy from the local environment by virtue of cell-intrinsic programs, which many viewed as validating the hypothesis that cell fate determination is manifested by irreversible progression down a lineage trajectory (Weismann, 1893), an idea so attractive that it was still regarded by some at the turn of the 21st century as dogma, despite continual challenges demonstrating that this was at least sometimes not the case. An early such example was the discovery that transplantation of all or a subset of cells of the limb field, i.e. the future limb, to another part of an embryo would result in the formation of an ectopic limb, and that cells from other regions transplanted into the limb field could subsequently contribute to the limb (figure 8D; Harrison, 1918), arguing that progenitors exhibit some degree of plasticity - a fancy way of saying their potential may be greater than initially believed. (As Director Josef said in *GATTACA*, "No one exceeds their potential. If they did, it would mean we did not accurately gauge their potential in the first place.") Importantly, however, the limb field experiments only indicated that uncommitted cells were simply redirected to a limb fate downstream of their current position in the lineage. Enter studies of *Drosophila* imaginal discs, i.e. the developing adult appendages: whereas cells from leg discs transplanted into wing discs normally maintain their identity, a subset of these cells, when cultured prior to transplantation, switch to wing fates (reviewed in Maves and Schubiger, 2003). Though this phenomenon has been termed "transdetermination" (Hadorn, 1965), the fact that a period of culture is required implies rather that the dissected disc cells have actually undergone *de*differentiation before redifferentiating along an alternative lineage, and hence arguing that commitment can be, at least partially, reversible.

Direct evidence of such reversibility derives from studies of transgenic mice. Mammals are typically less amenable to regeneration than reptiles and invertebrates; consequently it has been comparatively difficult to identify *bona fide* cases of dedifferentiation into stem cells. Using lineage tracing, a recent study of the intestinal epithelium reported that transit-amplifying (TA) cells committed to the secretory lineage can dedifferentiate in order to repopulate the stem cell pool following its depletion (van Es et al., 2012). Commitment is a quality intrinsic to a cell (albeit one influenced by extrinsic factors), so of critical importance is whether these TA cells represent a distinct, more differentiated cell type or are still stem cells that have simply migrated to a different location from the known stem cell pool. Indeed, loss of contact with the stem cell niche has been shown to lead to differentiation of adult stem cells, laser ablation of the hermaphrodite nematode gonadal niche being the classical example (Kimble and White, 1981), and it has previously been argued that TA cells may, in fact, be a population of *bona fide* migratory stem cells that is depleted after a limited number of cell divisions as a result of stochastic selection between self-renewal and differentiation (Lander, 2011a). However, stem and TA cell transcriptome analysis revealed that the TA cells do not express stem cell markers, but more definitively, the *in vitro* conditions for stem cell propagation are insufficient to maintain the TA population, demonstrating that these groups represent functionally distinct cell types, and hence that dedifferentiation had, indeed, occurred (van Es et al., 2012). Subsequent studies of the lung airway epithelium found that selective ablation of basal stem cells also led to dedifferentiation of secretory cells (Tata et al., 2013).

Adult stem cells have roles in tissue homeostasis and repair, and are required for the lifetime of the organism, so reversible commitment could have conceivably evolved exclusively to ensure that these functions be met. Indeed, pioneering studies of preimplantation embryos indicated a progressive and irreversible loss of potency over the course of embryonic development, as elegantly revealed by the ability of single injected mouse cells to form chimaeras in host embryonic day (e) 4.5 blastocysts, such that ectodermal cells of the inner cell mass (ICM) contribute to all embryonic tis-

sues and concomitantly fail to contribute to most placental tissues, whereas primitive endoderm cells conversely contribute exclusively to the primitive endoderm of the placenta (Gardner and Rossant, 1979). Similarly, ectodermal cells of the e6.5-7.5 epiblast fail to contribute to chimaeras when injected into the e5.5 epiblast (Gardner et al., 1985). However, recent studies of mouse ICM-derived embryonic stem (ES) cells and epiblast stem cells (EpiSCs) indicate that reversion is a feature of embryonic development as well: initial studies of EpiSCs indicated that they could not be derived under conditions of ES cell propagation (Tesar et al., 2007), but spontaneous conversion from an epiblast- to ICM-like state was reported when EpiSCs were propagated under ES cell conditions (Bao et al., 2009). Subsequently, it was shown that a developmental intermediate cell type, IES cells, could be propagated under hybrid ES/EpiSC culture conditions, as well as under either ES or EpiSC culture conditions (Chang and Li, 2013). Thus, cell identities within a given lineage in both the embryo and adult appear to exist along a commitment continuum, which, provided that the appropriate intermediate states are transited, is fully reversible.

In demonstrating the ability to form an ectopic limb, Harrison's limb field experiments were also important to show that cells possess an intrinsic memory of their developmental history that is resistant to environmental variation, yet, conversely, the ability of other cells to assume limb identity when grafted in the limb field is demonstrative of the sensitivity of cells to extrinsic cues. Together, these data suggested that intrinsic programs regulate the competence of cells to respond to such cues. Interestingly, in many cases, the cells themselves produce their own signals to differentiate, as exemplified by the ability to maintain ES cells in the undifferentiated state by inhibition of such auto-synthesized signals (Kunath et al., 2007). Together with the facts that neither cultured blastocyst-stage embryos nor ES cells spontaneously dedifferentiate into totipotent cells, nor do they produce signals sufficient to maintain them in their current state but rather tend to differentiate until they become post-mitotic, these data argue that commitment is characterized by an equilibrium biased toward differentiation, i.e. development is inherently directional (a sensible strategy, given cells' raison d'être to generate a mature organism).

Lineage Topology

Dividing cells always generate two progeny, so the lineage topology of an organism is, in one sense, always binary; however, the identity of those progeny can vary according to a multitude of criteria, including (i) the products of cell divisions; (ii) degree of determinacy, i.e. the variance in daughter cell identity of a given progenitor of the lineage between organisms of the same species; (iii) whether the hierarchical structure of the lineage is typological or topographical (see below); and (iv) the competence of that progenitor to generate daughter cells of one type or another. As such variables are the product of the stem cell regulatory architecture, they are highly informative in understanding stem cell decision making, and it is to them that lineage topology refers.

i) Modes of Cell Division

Cell division can be (1) symmetric or (2) asymmetric, each of which can be further subdivided into two modes according to subtype relationship to the progenitor and to each other (figure 9A): (1a) in the proliferative mode, the progenitor self-renews to produce two daughter cells with the same identity as the mother cell, increasing the size of the stem cell pool; (2a) in the progenerative mode both daughter cells differentiate to the same new identity, depleting the stem cell pool; (1b) the conservative[12] mode results in one differentiated and one self-renewing daughter, conserving the stem cell pool; and (2b) the diversifying mode leads to two differentiated daughter cells with different identities from each other and depleting the stem cell pool. Interestingly, whereas deuterostomes such as vertebrates typically begin development in the proliferative mode and switch to other modes later in development, many

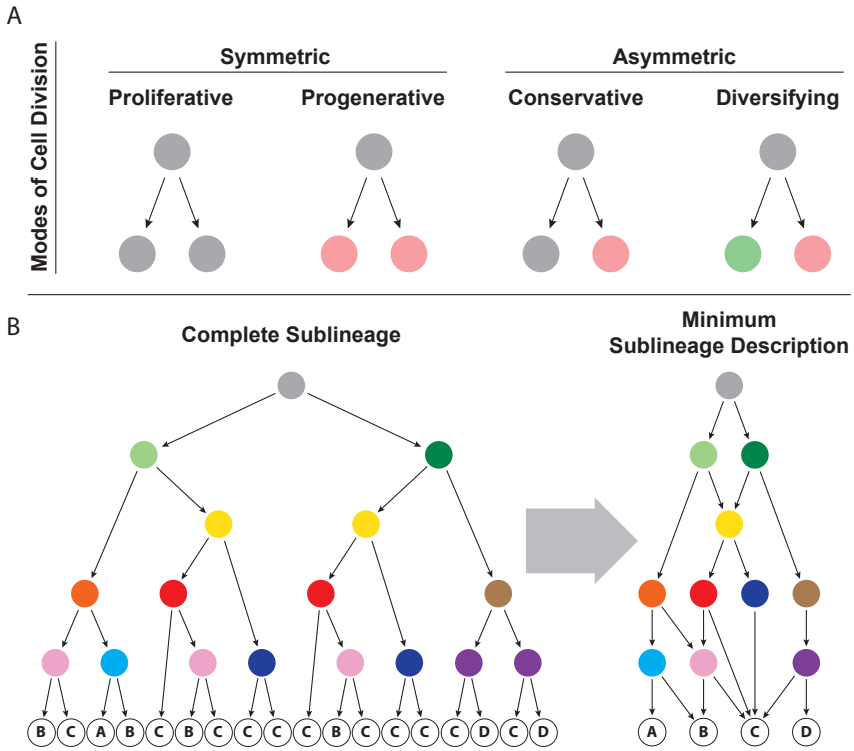

Figure 9. The building blocks of cell lineages.
(A) All cell lineages are constructed from a combination of just four modes of cell division, which are classed as symmetric or asymmetric, depending on whether both daughter cells assume the same fate. Both the proliferative and conservative modes result in stem cell self-renewal, though whereas the former increases the size of the stem cell pool, the latter maintains it, leading some to call it the 'stem cell' mode — misleadingly, as stem cells have been observed to divide by each of the four modes. Both the progenerative and diversifying modes both result in stem cell differentiation, but the daughters in the latter assume distinct fates from each other.
(B) Schematic illustrating the modular composition of a lineage in which four classes of terminal cell type (A-D) are generated. In this case, each yellow cell and its progeny constitutes a sublineage module that is used twice in the lineage and can be described as a series of rules: (1) a yellow cell always produces one red and one navy blue daughter cell; (2) the latter always generates two type C terminally differentiated cells, (3) the red cell always one C cell and one pink cell, (4) the latter of which goes on to generate one B and one C cell. By depicting this sublineage once, the complete sublineage can be abbreviated to a minimum sublineage description consisting of 11 rules vs. the 17 rules in the complete sublineage. Given the 17 cell divisions required to produce the 18 terminally differentiated cells, the lineage complexity is 0.65, compared to ~1.3 by chance. Generalized from the *C. elegans* sublineage portrayed in Azevedo et al., 2005.

protostomes, e.g. *C. elegans*, begin in the diversifying mode (see figure 8B) and subsequently switch to other modes (Stent, 1985). This may reflect the need to scale up cell numbers in larger organisms.

ii) Lineage Determinacy

Organisms face numerous selective pressures, each of which is a component of an equation that must satisfy the need to develop to a state of maximal probability of reproductive success as quickly as

possible while surviving in an environment with a given degree of variation. Depending on the strength of each of these variables, selection will therefore drive evolution to either (1) faster development, by decreasing organism size and/or variance of cell lineage topology, (2) increased size or specialization, and/or (3) increased adaptability through developmental selection. Obviously, an increase in (1) will tend to come at the expense of (2) or (3) and vice-versa. Each can be modulated by varying lineage determinacy (Snell-Rood, 2012), i.e. the degree to which the lineage is pre-defined such that the daughter cells of a given progenitor have an invariant or variant identity from one specimen of a species to another (Stent, 1985). Determinate lineage topology is the most intuitive, being the most evocative of purposeful design (albeit misleadingly so), and in this regard was first hypothesized over a century ago as a mechanism for Preformation (Weismann, 1893). Indeed, determinate development is essentially the mode for many simple metazoa such as worms, including *C. elegans* (figure 8B; Sulston et al., 1983). By strictly defining the order of cell type generation, determinate development can, where appropriately instituted, maximize the efficiency of time and energy expenditure through reduction in the number of cells required to construct an organism and precision in the position of terminal differentiation within the organism (Snell-Rood, 2012). Moreover, determinate development provides a mechanism for conflict mediation between cells of a multicellular organism (Michod and Nedelcu, 2003). On the other hand, determinate development is mosaic, i.e. the progeny of ablated embryonic cells are lost (Sulston et al., 1983), reducing their robustness in the face of environmental (and genetic) perturbation.

Developmental mechanisms permitting greater adaptability would have an increasing selective advantage as environmental variation increases. For example, in regulative systems such as the early mammalian embryo, cells (and any potential progeny) lost due to unexpected events can be replaced by the remaining cells (Stent, 1985). Regulative mechanisms of recovery are characteristic of indeterminate lineage topologies, and depend on cell equivalence, in which one of two cells that would normally be destined to adopt different fates assumes the identity of the other under abnormal circumstances (Stent, 1985). The discovery of such modes of development actually predated Weismann's hypothesis: fragments of Siphonophore[13] larvae were able to generate the entire adult organism (Haeckel, 1869), and separation of the cells of two-cell stage sea urchin embryos revealed that two complete sea urchins would develop (Driesch, 1892)[14]. In indeterminate lineages, progenitors from a pool of equivalent cells are selected on an *ad hoc*, random basis to differentiate to a given lineage (Stent, 1985). Cell lineages of species that undergo indeterminate development still have invariant topologies, but these are manifested at the level of progenitor pools, rather than individual cells, e.g. the invariant origin of the three primary germ layers exclusively from the mammalian blastocyst inner cell mass, as discussed above (Gardner and Rossant, 1979). The regulative nature of indeterminate lineages also offers opportunities for cell competition to eliminate unfit (i.e. mutated) cells during embryogenesis (e.g. Sancho et al., 2013), the importance of which is likely proportional to the size of the mature organism. On the other hand, cell competition is illustrative of the increased costs in time and resources that are intrinsic to indeterminate development. This is because developmental adaptability is an outcome of exploratory behavior, in which a range of options are generated in response to a given environmental variable, and the best response selected, i.e. trial-and-error[15]; its costs relative to potential benefits (the exploration-exploitation trade-off) are a principle evolutionary constraint on indeterminate development (Snell-Rood, 2012). It is worth noting that, while determinate lineages can be found in many small, simple organisms, such lineages have deviated substantially from the indeterminate lineages of our common ancestors, underscoring the fact that all extant organisms, whether the humble *C. elegans* or not-so-humble *H. sapiens*, represent nature's latest advancements.

iii) Lineage Topology Hierarchy

Lineages may be hierarchically organized to favor either typologic or topographic topology, in which cells of the same or similar type are generated either at the same site from a common progenitor pool or based on their final position in the mature organism, respectively (Stent, 1985). This could be imagined to have major implications for the underlying regulatory program, as the network of determinants of cell identity could conceivably be rather simple for a small organism with exclusively typologic hierarchy. However, the flip-side of typologic hierarchy is the need for tissue rearrangement, i.e. cell migration, in order for cells to arrive in their appropriate final locations following terminal differentiation, and this requires its own complex regulatory network. Each organism must therefore evolve a suitable balance between these conflicting aims, taking whatever advantages present themselves. By way of example, motor neurons are generated as a pool along the entire anterior-posterior (AP) axis of the ventral neural tube, but their target cells are muscles in the body which can be a meter away in humans, whereas the MNs themselves are innervated by interneurons in the central nervous system. Their generation at the edge of the nervous system at the AP position closest to their targets is therefore a compromise between efficient nervous control and efficient communication with target muscle fibers, and is thus a combination of typological and topographical hierarchy. By contrast, all the lymphocytes producing a given antibody species arise from a single cell, only to be dispersed around the body (Stent, 1985); however, because those cells are transported in the lymphatic vessels, the distribution process is not costly, so typological lineage organization is the most efficient option.

The *C. elegans* lineage hierarchy is largely topographically organized (Stent, 1985; Sulston et al., 1983), such that, for example, the last common ancestral cell of all of its neurons is the zygote (figure 8B; Sulston et al., 1983). This led to the suggestion that its lineage is complex (Sulston et al., 1983); however, subsequent analyses of determinate lineages from several species, including *C. elegans*, identified sublineage modules that are reiteratively deployed in the lineage (Azevedo et al., 2005). This permitted the lineage description to be abbreviated such that each module represents a rule that is described only once[16] (figure 9B). By calculating the number of rules as a proportion of the number of cell divisions, these biological lineages were found to be substantially simpler than would be expected by chance[17] (Azevedo et al., 2005). Nevertheless, simulations indicated that the lineages under examination could theoretically have evolved to be simpler, implying that additional selective pressures constrain their simplification. Topography appears to prevent this, owing to the fact that lineage itself plays a causative role in which cells instruct each other through direct contacts during embryogenesis, precluding the removal or rearrangement of many, if not most, cells of the lineage tree in the absence of additional mechanisms to coordinate cell migration (at the very least). The simplest lineages are therefore mutationally inaccessible (Azevedo et al., 2005), or as Coelho wrote, "Choosing one path meant abandoning others."[18]

iv) Competence Topology

Assessments of lineage topology in terms of the *realized* output of each cell division under normal conditions, while informative, have limits in their utility, particularly to understand indeterminate development in which the lineage is less stringently regulated and has a more modest causative role. However, lineage topology can also be described in terms of progenitor competence leading to commitment, i.e. the number of identities a progenitor can potentially assume when it divides. It has been suggested that cell fate decisions in "higher" metazoans are universally ternary[19] in nature, the stem cell choosing either to self-renew or differentiate into one (or both) of two downstream fates (Zhou and Huang, 2011); however, an abundance of evidence indicates that, while the differentiation of certain progenitor types, e.g. common myeloid progenitors, is governed by ternary topology, this is far from

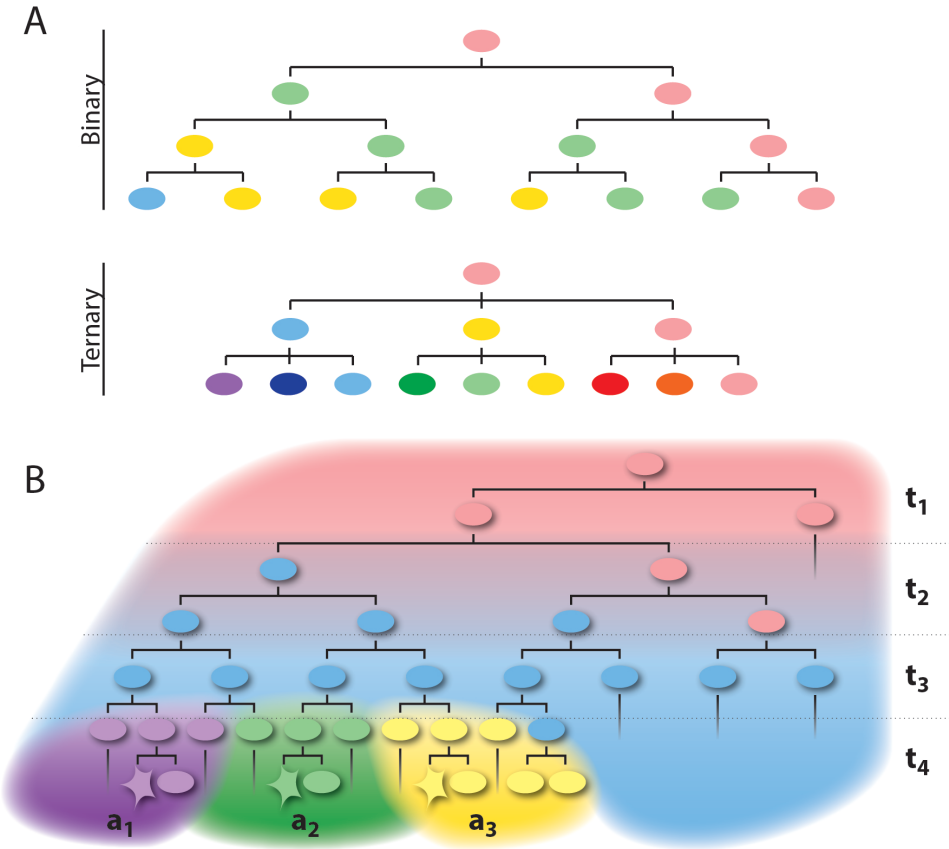

Figure 10. Lineage competence topology.
(A) Schematic of two hypothetical lineage competence trees in which each cell faces a binary or ternary fate decision upon cell division. One of these options is to self-renew, and, depending on its regulatory parameters, the stem cell may be competent to generate one or more alternative fates (upper and lower lineages, respectively). This menu of options is not to be confused with asymmetric cell divisions: in principle, each cell may choose any of the four modes of division, which may produce daughters fated to the same or distinct fates. While increasing the competence of the stem cell from binary to ternary (or greater) increases cell flexibility to respond to environmental cues, it may come at a cost of a corresponding increase in regulatory complexity, and consequently would be more difficult to evolve. Of course, the fact that it has not evolved extensively in lineage trees suggests that it is not beneficial under most circumstances, else it would have.
(B) Schematic of a hypothetical spatiotemporal execution of a unary-binary competence lineage tree as might be observed in vertebrate embryogenesis. Area shading indicates competence $\mathbf{t_1}$: The stem cell is unicompetent and divides in the proliferative mode. $\mathbf{t_2}$: A transitional period in which a switch to bi-competence leads the stem cell (red) to choose between one of two distinct identities through self-renewal or differentiation to stem cells with more restricted potential (blue cells). Once differentiated, the cell will not return to the prior fate under normal conditions. The stem cell may divide in the proliferative or conservative mode. $\mathbf{t_3}$: Differentiated progeny undergo self-renewal in the proliferative cell division mode and any remaining red cells undergo differentiation in the progenerative division mode to blue cells. $\mathbf{t_4}$: Spatial compartmentalization leads to a switch in competence based on cell position within the tissue. Blue cells differentiate as they are incorporated into one of areas $\mathbf{a_{1\text{-}3}}$ and in subsequent divisions may choose whether to self-renew or terminally differentiate (star-shaped cells) via any of the proliferative, progenerative, or conservative division modes.

universal: for example, while murine neural progenitors are multipotent, around midgestation they are faced with a binary decision of self-renewal or terminal neuronal differentiation and only subsequently undergo a competence switch to a program of glial differentiation (Kohwi and Doe, 2013). Similarly, the differentiation of pluripotent ICM cells to primitive endoderm and the transition from ICM to epiblast cells are *temporally* segregated binary decisions (Gardner, 1983), and the differentiation of epiblast tissue to ectoderm and mesendoderm are *spatially* determined binary decisions (Beddington and Robertson, 1999). The prevalence of such binary decisions supports the notion that they may be more readily evolvable than those offering multiple alternatives simultaneously. Thus, lineage topology in indeterminate development does not follow a strict, universal structure, although it is tends to consist of relatively simple unary or binary decisions regulated in a spatiotemporally manner (figure 10).

THE MOLECULAR BASIS OF CELL DIVERSIFICATION

A priori, stem cells could be viewed as containing a complete repertoire of properties of an organism that are progressively whittled down as cells follow distinct, determinate developmental trajectories (figure 11; Weismann, 1893). They could be also be imagined as more-or-less clean slates, acquiring new traits through differentiation in response to environmental cues (Zipori, 2004). Alternatively, they could possess many traits at low levels, subsets of which would be selected and amplified while others repressed in response to environmental stimuli (Zipori, 2004). A further possibility is stem cells possess some properties that are shared by differentiated cells and each possesses some that are not shared by the other, at a variety of levels, influenced by their environments. Importantly, in each of these scenarios, cell identity is a collection of traits, and context-specific functions of cells therefore imparted by modifying that collection of traits.

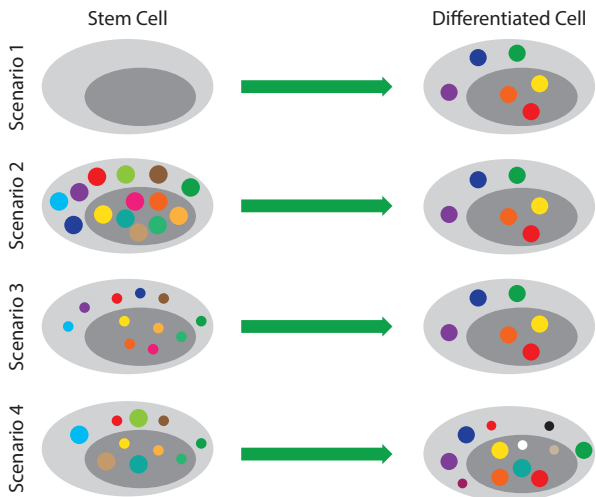

Figure 11. Four models of the route to differentiation, in each of which the traits present in the cell define its identity, and these are influenced by the surrounding environment. In scenario 1, this leads to the acquisition of traits over the course of differentiation, whereas in scenarios 2 and 3, differentiation is manifested in the loss of most traits and retention of a defined subset. In scenario 3, all traits are initially present at low levels and selected traits amplified while the rest are repressed. In scenario 4, traits are present at variable levels in both cell states, but some are lost and others gained over the course of differentiation. Scenarios 1 and 3 previously defined by Zipori (2004), scenario 2 by Weismann (1893).

Around the turn of the twentieth century the Atomistic proposal was made that these traits comprise a complete set of heritable cell fate determinants, each of which uniquely specifies one cell type, and this set of determinants was thought to be exclusively passed between cells of the germline. By contrast, somatic cells would receive only the subset of these determinants necessary to perform their specific tasks, and these would be progressively reduced the further they differentiated (Weismann, 1893). Thus was born the Master Regulator: an enduring, if overly simplistic, concept discussed further below. Though the concept of a germline has been empirically validated, a logical inconsistency in the overall theory was that each germ cell was considered to have only one set of determinants to pass along, so passing a subset of them to somatic cells would deplete the pool needed to create a new organism (Gilbert, 2014). Proof that somatic cells contain all necessary information required for the development of a complete organism came in the Nobel Prize-winning cloning of a frog by somatic cell nuclear transfer (Gurdon et al., 1958).

Today, it is well known that, as Mendel proposed, the basis for heritability of any trait is genetic[20] (Griffiths, 2000). Work over the better part of the century after Mendel converged on the familiar consensus, now dogma, that genes are encoded by DNA, which, in eukaryotes, primarily resides in the cell nucleus, and transcribed into RNA, which may be subsequently translated into protein

(Alberts, 2015). These findings have led to the ubiquitous analogy that the genome is a blueprint that defines the properties of the organism, such that cell identity, at a molecular level, is defined intrinsically by the sum expression of genes, called the transcriptome and proteome. It follows that any context-specific functions of the cell must be regulated at the level of transcription, translation, or post-translational modification of protein function, examples of all of which, of course, are widespread within a cell (Alberts, 2015). The prevalence of mechanisms that control each of these processes will generally be dependent on the importance of responsiveness weighed against the need to prevent unnecessary energy expenditure and potentially toxic interactions, as well as the extent to which context-specific functions are co-regulated. Nevertheless, it is not glaringly obvious that any of these mechanisms are principle determinants of the degree of cell diversity (and hence organism complexity) observed between species.

This is because from a perspective of purposeful design, which is how man tends to reason, a more obvious means of achieving greater diversity is to increase the number of genes in the genome, but while gene number is a theoretical constraint, it does not seem to be the key driver of complexity, as, for example, both the human and *C. elegans* genomes contain ~20,000 genes (Consortium, 1998; International Human Genome Sequencing, 2004). This figure prompted much navel-gazing about how *Homo sapiens* came to be so darned special (Pennisi, 2012), but was (particularly with the benefit of hindsight) rather an odd way of looking at it: even to simplify gene expression as a binary quality, consider that 20,000 genes provides $2^{20,000}$ distinct potential expression states — iterative orders of magnitude greater than the number of atoms in the observable universe, let alone the $\sim 10^{14}$ cells in the adult human body (Lodish, 2013). As the saying goes, it's not the size that counts; it's how you use it. In scientific parlance, this concept is termed pleiotropy: the assignment of many otherwise unrelated functions to single genes (Gilbert, 2014). From an evolutionary perspective, this makes a great deal of sense, as it is easier to recruit available tools for additional tasks than to evolve them *de novo*.

The Regulatory Architecture of the Genome

Though various explanations such as protein processing have been mooted that likely contribute to cell diversity to varying degrees, the most compelling relates to genome size: at ~3 gigabase pairs, the human genome is over an order of magnitude larger than that of *C. elegans* (Consortium, 1998; International Human Genome Sequencing, 2004). In mammals, ~99% of the genome is non-coding, leading many to label it 'junk' DNA until large-scale analysis indicated that at least 80% of it had some biochemical function (Consortium, 2012; Maher, 2012); because of the practical constraints of the analysis, the actual figure is likely substantially higher (Consortium, 2012). This chimes with the fact that protein-coding sequences themselves do not contain sufficient information to drive their own transcription, but rely on *cis*-acting elements, as first revealed in a ß-thalassemia patient harboring a deletion mutation outside the *Hbb* (ß-globin) coding region, demonstrating that a critical function of non-coding DNA is to regulate gene expression (Van der Ploeg et al., 1980). Subsequent analyses of non-coding mutations led to the identification of distinct categories of *cis*-regulatory architecture (reviewed in Lenhard et al., 2012), namely the ~100 base pair (bp) minimal or core promoter that spans the transcription start site (TSS) and recruits the basal transcriptional machinery; larger elements called proximal promoters always located immediately upstream of the core promoter; and distal elements that may be located either upstream or downstream of the TSS, ~120 kilobases (kb) away on average but in some instances over a megabase (Mb) away (de Laat and Duboule, 2013). Studies of the *Hbb* gene and viral DNA elements outlined the principles that core promoters do not efficiently drive transcription, which is regulated by enhancers that drive gene expression independently of sense/antisense orientation

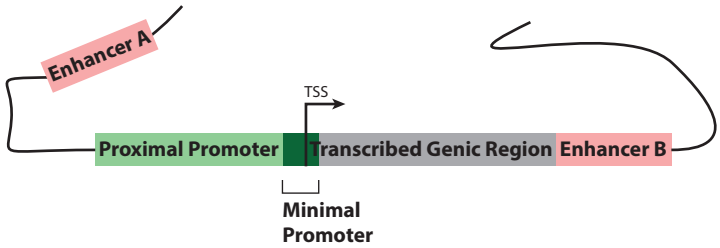

Figure 12. Basic organization of *cis*-Regulatory Elements. The minimal promoter contains sequences required but not sufficient for recruitment of the basal transcriptional machinery. Such information can is contained within proximal promoter and distal enhancer sequences, which coordinately direct cell type-specific gene expression. In contrast to promoters, enhancers can function in either orientation, and over short and long distances. *TSS*: transcription start site

(Banerji et al., 1981). In the fruitfly, *Drosophila melanogaster*, large-scale screening of genomic DNA for transcription-driving activity indicates that the majority of regulatory elements do not overlap with coding regions (Arnold et al., 2013). Moreover, in a large-scale "enhancer trap" screen in which a minimal promoter and reporter gene were transposed into the mouse genome, 60% of the insertion sites resulted in spatially restricted reporter expression around midgestation (Ruf et al., 2011). Interestingly, many enhancers of developmentally expressed genes appear to act redundantly, such that loss of one enhancer does not affect development under normal conditions, but under abnormal conditions, the remaining 'shadow' enhancer fails to properly regulate gene expression, indicating a requirement of redundancy for robust gene regulation (reviewed in Spitz and Furlong, 2012). Collectively, these studies indicate that a massive proportion of the vertebrate genome far exceeding the coding portion serves to regulate gene expression in a spatiotemporally precise manner, implying that the physiological and behavioural complexity of *H. sapiens* is due primarily to alterations of the gene regulatory landscape[21] that lead to repurposing of genes, thereby increasing their functional output without without necessarily needing to alter their biochemistry, which would mostly be detrimental to pre-existing functions (de Laat and Duboule, 2013). An important question, however, is how enhancers are able to act over typically long distances, which requires an understanding of how DNA is organized in the nucleus.

Chromatin

One need only think of tangled Christmas tree lights to appreciate that chromosomes, the single-molecule strings of double-stranded DNA that extend many Mb in length and on which hundreds of genes are arrayed, would quickly become an unwieldy mess without a molecular scaffold to keep them ordered. The units of this scaffold are histone proteins, and histone-bound DNA is called a nucleosome or, at the genomic scale, chromatin (Alberts, 2015).

Chromatin Modification and the Epigenetic Regulation of Transcription

Decades of research have demonstrated that chromatin may be covalently modified, critically altering gene expression, an effect termed epigenetic regulation. Studies of chromatin composition identified methylated cytosine residues (Hotchkiss, 1948), which were specifically found adjacent to guanine residues (Doskocil and Sorm, 1962) and subsequently shown to specifically arise by postsynthetic modification (Scarano et al., 1965). Eventually, gene activation during the initial stages of development was found to be accompanied by loss of DNA methylation (Bird et al., 1981), and methylation shown to be required for maintenance of gene silencing (Compere and Palmiter, 1981).

Moreover, regions of non-methylated high GC content, termed CpG islands, were found to be over-represented in the vicinity of active genes as well as enhancers (Bird et al., 1985; reviewed in Lenhard et al., 2012). Unmodified histones also generally inhibit transcription by limiting accessibility for the transcriptional machinery (Allfrey et al., 1963), and analysis of nucleosome positioning around the *Hbb* promoter in distinct cell types using DNA footprinting, in which naked but not protein-bound DNA is enzymatically or chemically degraded, found that nucleosome repositioning precedes *Hbb* gene activation, although this did not establish causality (Benezra et al., 1986). Whereas DNA itself may only be methylated, myriad histone modifications have been identified that influence

	Enhancer States			
	Active	**Primed**	**Poised**	**Inactive**
eRNA transcription	x			
H3K27ac	x			
H3K4me1/H3K4me2	x	x	x	
H3K27me3			x	x

transcription in distinct ways. Histone acetylation was the first to be identified and proposed to promote transcription by reducing histone affinity for DNA (Allfrey et al., 1964). A number of modifications of the H3 histone subunit have been shown to be associated with gene transcriptional status, as well as marking enhancers and promoters. In particular, acetylation of lysine 27 (H3K27ac) is a mark of active promoters and enhancers, whereas trimethylation of lysine 27 (H3K27me3) is associated with repression; methylation of lysine 4 marks non-silenced promoters and enhancers (H3K4me), and in the absence of H3K27 modifications such enhancers are said to be "primed", whereas when accompanied by H3K27me3 they are termed "poised", reflecting a higher relative threshold required to activate transcription (reviewed in Heinz et al., 2015; Lenhard et al., 2012). Similarly, at "bivalent" promoters, the presence of both H3K4me and H3K27me3 denotes a poised transcriptional state (Bernstein et al., 2006). Although H3K4me has not been shown to directly influence transcription, it is thought to influence the ability to methylate and maintain methylation of H3K27 (Zentner and Henikoff, 2013), and its maintenance under certain circumstances following gene repression has been proposed as a mechanism of cellular memory (Lenhard et al., 2012). That said, recent studies indicate that only a subset of active elements are poised in the parental cell type, arguing that this step is not necessarily required for activation (Rada-Iglesias et al., 2011). Curiously, active enhancers themselves have recently been shown to be transcribed to produce enhancer RNAs (eRNAs; Kim et al., 2010), and this appears to be important for target gene activation (reviewed in Heinz et al., 2015). Given that many eRNAs evidently do not to have sequence-specific functions, it has been proposed that enhancer transcription could facilitate gene activation via transcription-dependent nucleosome remodeling (Heinz et al., 2015).

	Promoter Class	
	type I: tissue-specific	**type III: developmental**
Enhancer Interactions	in vicinity	many, often long-range
Possible poised state	no	yes
Repression	DNA methylation	H3K27me3
CpG Islands	low	large

Studies of chromatin features have been instrumental in classifying promoter structure-function relationships, identifying three principle types (reviewed in Lenhard et al., 2012), of which types I and III are of particular relevance here. Type I promoters are typically associated with genes expressed in a tissue-specific manner, particularly terminally differentiated cell types, are predominantly regulated by nearby enhancers and generally are either active or repressed, and are preferentially silenced via DNA methylation rather than histone methylation (Xie et al., 2013). By contrast, type III

promoters are typically associated with developmentally expressed genes and tend to be situated around large CpG islands that can be DNA methylated but more commonly are silenced via H3K27me3 (Xie et al., 2013). They are commonly found to be poised in certain contexts (e.g. Bernstein et al., 2006) and are typically regulated by many distal elements, consistent with their reiterative deployment in diverse developmental processes.

Chromatin Interactions Over Long Distances

Epigenetic modifications can lead to more compacted (closed) or relaxed (open) conformations in a site-specific manner as well as globally. Open conformations are characterized by DNase I hypersensitivity, i.e. degradation due to a lack of insulation by histones (Alberts, 2015). As one would intuit, actively transcribed genes and active enhancers are associated with such regions, whereas silenced genes tend to be associated with closed chromatin, implying a dynamic quality of chromatin topology during development (reviewed in Stamatoyannopoulos, 2012). Such dynamic reorganization was shown to occur over the course of the yeast cell cycle using chromatin conformation capture (3C) techniques, in which cross-linked DNA is restriction-digested and ligated such that DNA fragments are joined based preferentially on close physical proximity in the nucleus over linear order on the chromosome, permitting identification of long-range interactions with a given locus by PCR (Dekker et al., 2002).

These findings suggest two principle alternative models of enhancer regulation of transcription: in contactless models, enhancer activation would result in remodeling of chromatin architecture, e.g. by displacing nucleosomes via a chain reaction, whereas in contact models, interaction between the enhancer and promoter would stabilize binding of the transcriptional machinery (Carter et al., 2002). Support for the contact model was promptly provided by 3C studies of the *Hbb* locus, in which it was demonstrated that chromatin looping can bring enhancers into direct contact with promoters in a cell type-specific manner, and this looping is coincident with a transcriptionally active state of *Hbb* (Tolhuis et al., 2002). A notable caveat for students of gene regulation, studies indicate that only a small fraction of long-range interactions with enhancers involve the nearest gene (Sanyal et al., 2012). Consistent with the idea that chromatin remodeling is a prerequisite for transcription, subsequent studies have demonstrated that looping is required for transcription of many genes, but not necessarily indicative of a transcriptionally active state (figure 13; de Laat and Duboule, 2013). Moreover, using Hi-C, a 3C-based approach in which DNA is labelled prior to ligation to enable unbiased purification of labelled fragments for sequencing, it was shown that open and closed chromatin occupy distinct nuclear compartments, with silenced genes typically localized closer to the nuclear lamina (figure 14; Lieberman-Aiden et al., 2009). As recognition has grown of the importance of looping for transcriptional control, contactless models of enhancer activity have largely fallen by the wayside, but, as the saying goes, absence of evidence is not necessarily evidence of absence.

Interestingly, some long-range contacts appear to be invariant across cell types (and species), demarcating Mb-scale local chromatin territories, termed topologically associated domains (Dixon et al., 2012), and it is only within each domain that looping varies across cell types (figure 14; Jin et al., 2013). A key outcome of this organization is that functionally unrelated genes within a given domain tend to be co-regulated (the so-called 'bystander' effect), while genes within distinct domains are regulated independently of the topology of one another. Importantly, domain-constrained organization also provides for context-specific gene regulation by distinct enhancers, such that many tissue-specific enhancers may collectively produce an essentially ubiquitous expression pattern (Kieffer-Kwon et al., 2013).

Transcriptional Regulation of Gene Expression

Although the spatial organization of the genome and DNA sequence are critical determinants of gene regulation, DNA does not transcribe itself, so this must be coordinated by gene regulatory networks. Fundamentally, there are two potential ways in which TFs could regulate identity: they could selectively activate a given gene expression programs in each cell type, or they could actively suppress only undesired programs, resulting in derepression of the desired program of cell identity. To varying degrees, these alternatives are combined in stem cells. (Why settle for cake when you can have it with ice cream?) Whereas in bacteria, transcription initiation and elongation by RNA polymerase is an energetically favorable process (Cooper and Hausman, 2013), the presence of histones in eukaryotes renders transcription energetically unfavorable (reviewed in Bell et al., 2011). However, given the more complex life cycles of eukaryotes (and of multicellular eukaryotes in particular), this higher threshold for transcription is likely to be an advantage, as genes with context-specific roles must be efficiently repressed in many cases so as not to disrupt the normal functions associated with a given cell type.[22] When few in number, such functions are most easily regulated by dedicated *trans*-acting[23] transcription factors (TF) by virtue of promoter-specific activity, as is the case in *E. coli* (Cooper and Hausman, 2013). However, commensurate with increasing gene regulatory network complexity, such TFs would have been progressively co-opted to regulate additional functions by simple evolutionary mechanisms (detailed below).

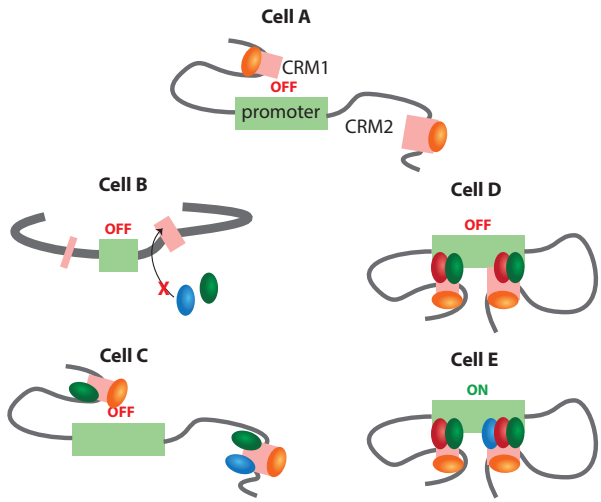

Figure 13. Regulation of enhancer looping and gene expression. The context-specific activity of a hypothetical gene regulated by two distal enhancers (CRM1-2) is shown. In cell A, a pioneer transcription factor (TF; orange oval) is expressed and binds each CRM, leading to an open chromatin conformation, but other activator TFs are not expressed so the enhancers are not in the vicinity of the promoter and the gene is silent. In cell B, the pioneer TF is not bound and chromatin is in a closed conformation that is inaccessible to expressed enhancer-binding TFs (blue and green ovals). In cell C, the pioneer TF is bound to enhancers, allowing binding of additional TFs, but there is no contact with the promoter, as in cell A. In cell D, a DNA-bending TF (red) is bound to both enhancers, in addition to the pioneer and an additional TF, but a critical activator (blue) is not expressed, and the gene remains silent. Only in cell E are all components present leading to open chromatin, enhancer looping, and recruitment of the basal transcriptional apparatus.

Transcription Factors

Sequence-specific TFs were first proposed to operate in *E. coli* to regulate enzymatic production by repression (Jacob and Monod, 1961), resulting in a Nobel prize following the identification of the corresponding repressor, *LacI* (Gilbert and Muller-Hill, 1966), and though it was suggested that repression would be the principle mode of sequence-specific transcriptional regulation (Jacob and Monod, 1961), there was little reason to rule out the existence of sequence-specific transcriptional activators. Indeed, the discovery of the first eukaryotic context-specific TF, SP1, in partially purified crude cell extracts provided proof of concept that selective target gene activation plays a role in transcriptional regulation in eukaryotes (Dynan and Tjian, 1983a). The SP1-binding site was also the first to be defined in eukaryotes, by mutagenesis of promoter sequences of a known target gene (Dynan and Tjian, 1983b), and these experiments made it possible to purify SP1 using synthetic oligonucleotides of that sequence cross-linked to sepharose beadas (Kadonaga and Tjian, 1986). Studies of LacI showed that context-specific transcription factors recognize and bind a range of oligomeric[24] DNA sequences of length that vary from an idealized consensus, such that TF binding affinity decreases as the sequence deviates from the consensus (Riggs et al., 1972). Importantly, as TF-DNA interactions occur via comparatively weak electrostatic and van der Waals' forces (e.g. Dragan et al., 2006), they should be considered *metastable* rather than truly stable, the implication being that binding of a given TF molecule is transient, even for high-affinity sites. Thus, binding of individual TF molecules to functional recognition sites lasts from a few to a score seconds (reviewed in Biggin, 2011). Mathematical modeling of the rate of binding of LacI to its recognition site indicated that it is much too frequent to be a simple function of TF diffusion in three-dimensional space, leading to a one-dimensional model of facilitated diffusion, i.e. that TFs sample potential binding sites by sliding along DNA (Riggs et al., 1970a; Riggs et al., 1970b). More recent studies of such behavior has led to a revised model combining one- and three-dimensional diffusion in which TFs slide for ~100bp without encountering a binding site before dissociating (see Halford and Marko, 2004 and references therein).

Although repressors conceivably could simply directly compete with the RNA polymerase-containing basal transcriptional machinery for DNA binding, this would obviously not be a suitable strategy for activators, implying that other mechanisms must exist, one of which could be direct interaction with the basal machinery to stabilize its assembly at the promoter, raising the question of how protein-protein and protein-DNA interactions are coordinated by the TF. Studies of the yeast transcriptional activator GAL4 provided early evidence that eukaryotic TFs are modular, as the isolated region of the protein responsible for DNA binding was unable to activate its target promoter, and, indeed, had a modest repressive effect (Keegan et al., 1986). Moreover, a chimeric protein in which the DNA-binding domain (DBD) of GAL4 was substituted by that of the *E. coli* repressor LexA was able to regulate transcription from a transgenic LexA-binding site, but rather than repressing transcription, it was activated, implying that the transactivation and transrepression domains interact with the basal transcriptional machinery in distinct ways and/or by recruiting distinct cofactors to regulate gene expression (Brent and Ptashne, 1985). Subsequent studies of truncated GAL4 variants inferred a specific interaction between the transactivation domain and the basal transcriptional machinery from DNase I footprint analysis of a synthetic promoter in the presence of different combinations of these factors (Horikoshi et al., 1988). Among the first repressor TFs to be identified in eukaryotes was *En*, which, by footprint analysis, was found to compete with the core transcriptional apparatus for DNA binding (Ohkuma et al., 1990). Similarly to manipulations of GAL4, a chimeric protein in which the En DBD was replaced by that of Ftz, an activator that recognizes a similar binding site as En, continued to strongly repress transcription (Jaynes and O'Farrell, 1991). Seemingly mutually incompatible domains can also coexist in the same protein, e.g. the amino-terminal transrepression domain and carboxy-terminal transactivation domain in Gli2 and Gli3 TFs, in which the activity of the transrepression do-

main is inhibited by the latter in the full-length protein, but revealed upon partial proteolysis of the transactivation domain (Pan et al., 2006; Wang et al., 2000).

While domain-based structural organization is an ubiquitous feature of the proteome, an additional level of modularity that plays a critical role in many protein-protein interactions is the linear motif,

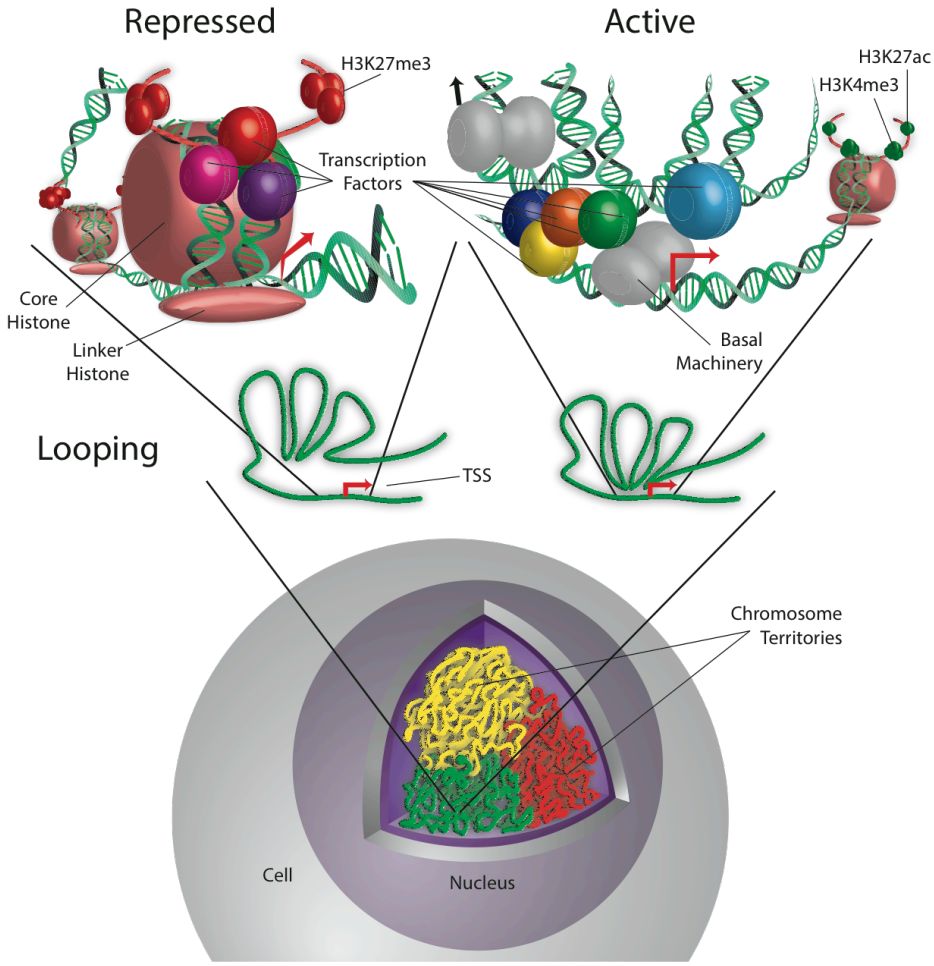

Figure 14. Chromatin organization and transcriptional regulation. Bottom: view of the nuclear territories of three chromosomes (red, yellow, green) in a given cell type. Middle: a hypothetical topologically associated domain within one chromosome that includes a gene that is transcribed from the indicated transcriptional start site (TSS) when distal elements are in the vicinity of the promoter (right), and this gene is otherwise transcriptionally inactive (left). Top left: in the repressed state, this TSS is inaccessible to the basal transcriptional machinery due to an overlapping nucleosome which is trimethylated at H3K27 as a result of binding by repressor TFs (red, pink, and purple spheres). Moreover, distal enhancers are not in contact with the promoter region. Top right: activator TFs (dark blue, yellow, orange, green, and light blue) have resulted in nucleosome repositioning, interaction of transcribed distal enhancers with the promoter, and recruitment of the basal transcriptional machinery (grey) to the promoter. Remaining histones near the TSS are acetylated at H3K27 and trimethylated at H3K4. Coactivators and corepressors are not included for simplicity.

a short peptide stretch characterized by weak affinity for protein partners — a result of its short length (reviewed in Neduva and Russell, 2005). Many such motifs exist in the genome and play crucial roles in diverse processes such as localization to specific cellular compartments. Many linear motifs are relatively weakly conserved in orthologous proteins but nevertheless are often found in many unrelated genes within a given genome and across the genomes of distantly related species, implying that their presence is determined by convergent evolutionary mechanisms. Linear motifs appear to play a critical role in detemining the sequence-specificity of some TFs. For example, studies of the Hox family of TFs have demonstrated the presence of a hexapeptide linear motif that mediates protein-protein interactions with Pbx TFs (Shanmugam et al., 1997), and such interactions are required to reveal "latent" DNA-binding specificity of Hox paralogues, which otherwise recognize the same DNA-binding site (Slattery et al., 2011).

A general mechanism analogous to the coupling of DBDs and protein-protein interaction domains in a single gene is the recruitment by TFs of cofactors: middle men with no intrinsic DNA-binding capability that serve as co-activators or co-repressors. Direct evidence for such cofactors was provided by studies of the herpes simplex virus transcription factor VP16, which had been found capable of inducing viral gene expression via specific DNA sequences, but could not actually bind those sequences (Marsden et al., 1987). Elctromobility gel shift assays, in which radiolabelled oligonucleotides run on a gel migrate more slowly when bound by proteins, indicated that the TF Oct1 could bind these sequences and VP16 (Gerster and Roeder, 1988), and using affinity chromatography, it was shown that VP16 could also interact with an immobilized component of the basal transcriptional machinery, suggesting that it recruits the transcriptional apparatus to target genes via Oct1 (Stringer et al., 1990). As a result of their potent gene regulatory activities, both the VP16 transactivation and the En repressor (EnR) domains have been extensively used to generate chimeric proteins in order to determine whether TF-mediated functions are a result of transcriptional activation or repression (e.g. **Paper II**).

While interactions with the basal transcriptional apparatus clearly play an important part in gene regulation, it is not clear whether or how this could relate to the chromatin topology dynamics discussed above, and it is therefore not surprising that more direct mechanisms have been identified that regulate chromatin accessibility. Indeed, although early studies demonstrated that many critical aspects of TF function is mediated via protein-protein interactions that do not involve the DBD, studies of GAL4 demonstrated that interaction with nucleosomes is directly mediated by the GAL4 DBD that formed a complex with histones and DNA resulting in nucleosome displacement (Workman and Kingston, 1992). TFs that can execute such displacement are called pioneer TFs, as they initiate a cascade of chromatin remodeling events that can (but do not necessarily) lead to gene activation (reviewed in Iwafuchi-Doi and Zaret, 2014). Foxa-class TFs are well-characterized examples, the DBDs of which resemble that of the linker histone H1 with which they are thought to compete for DNA binding in both open and closed chromatin. Foxa TFs subsequently directly interact with core histones to establish a local region of open chromatin. Given these activities, it is, perhaps, not surprising that the TFs Pou5f1 (Oct4), Sox2, and Klf4, which cooperatively mediate reprogramming of somatic cells to induced pluripotent stem cells, are pioneer TFs (Iwafuchi-Doi and Zaret, 2014). Extending the concept of pioneering, it has shown that, within a given lineage, sequentially expressed paralogous pioneer TFs bind initially silent enhancers, resulting in bivalent histone marks, of genes subsequently expressed in specific sublineages, e.g. binding of Sox2 in ES cells at enhancers of Sox3-expressing neural progenitor markers, to which the neural-specific TF Sox3 can bind upon neural differentiation (Bergsland et al., 2011). Sox proteins are notable for (but by no means unique in) their ability to bend DNA, binding in the minor groove (in contrast to most TFs that require the greater accessibility afforded by the major groove) and intercalating with DNA to stabilize the dis-

torted structure, suggesting that Sox proteins could directly contribute to chromatin looping (reviewed in Malarkey and Churchill, 2012).

Just as cofactors can extend the regulatory potential of TFs by mediating interactions with the basal transcriptional machinery, so too can they regulate chromatin accessibility and remodelling, coupling specific gene regulatory elements bound by TFs to non-specific regulators of gene expression and chromatin architecture. Studies of the Tle family of corepressors and its orthologs in flies and yeast have shown that disruption of both DNA binding of the basal transcriptional apparatus as well as its interactions with TFs are initiated by recruitment of Tle proteins by TFs, leading to direct core histone-Tle interactions, recruitment of histone deacetylases (HDACs) to chromatin, and ultimately to deactivation of regulatory elements (Turki-Judeh and Courey, 2012). Here, the details become rather murky, and two models have been proposed. The first relies on the well-established ability of Tles to multimerize, which has been shown *in vitro* to follow initial HDAC recruitment and histone deacetylation. Multimerization would then lead to further HDAC recruitment over a wider range of chromosomes and consequently chromatin condensation (Sekiya and Zaret, 2007). However, mutants that are unable to self-associate are still able to repress some loci in a Tle-dependent manner, although the mutation is lethal nonetheless, suggesting that the multimerization step may only be required for repression of a subset of target genes (Jennings et al., 2008; Kaul et al., 2014).

Coactivators also play a crucial role in chromatin remodeling. The p300 histone acetyltransferase, for instance, interacts with hundreds of proteins, including both sequence-specific transcription factors and the basal transcriptional machinery, as well as mediating acetylation of histone H3 (Holmqvist and Mannervik, 2013). Strikingly, analyses of p300 binding in the developing nervous system have found it to be highly associated with active enhancers, albeit not exclusively so (Visel et al., 2009).

A key problem in understanding the role of a TF in the processes it regulates is to determine which genes are regulated directly and which indirectly, i.e. downstream of other TFs that are targets of the TF under examination. Great strides toward this have been made through the use of antibodies designed to recognize specific TFs, which can be used for chromatin immunoprecipitation (ChIP), in which protein-DNA complexes are reversibly cross-linked and affinity purified and the sequence of bound DNA to be determined (Gilmour and Lis, 1984). Variations of this method have been widely used to study genome-wide TF binding, leading to three (among many other) interesting observations: firstly, only a small percentage of the potential TFBSs found throughout the genome are typically bound in a given population (e.g. Cao et al., 2010); secondly, TF binding is only weakly correlated with expression of neighboring genes (e.g. Vokes et al., 2008); and thirdly, the positions of bound TFs and nucleosomes are highly anti-correlated, and this relationship is more predictive of TF binding than binding site affinity (reviewed in Spitz and Furlong, 2012). Numerous factors are likely to contribute to this general pattern of TF binding site occupancy, including (1) previously unappreciated roles of TFs in transcriptional repression; (2) the variable accessibility of chromatin from one cell type to another (Sanyal et al., 2012); (3) that only 7% of enhancers make physical contacts with the nearest gene in a given cell type, so enhancers may be regulating more distal genes (Sanyal et al., 2012); (4) roles of TFs in other processes, e.g. DNA repair (reviewed in Malewicz and Perlmann, 2014); (5) roles in chromatin remodeling such as pioneering and/or other mechanisms for priming enhancers/promoters that are co-regulated by additional TFs not expressed in that particular cell population (figure 13; Iwafuchi-Doi and Zaret, 2014); and (6) the related facts that TF binding kinetics are more predictive of activation than binding per se, as brief binding may not be sufficient to reorganize chromatin and/or stabilize transcriptional machinery (Lickwar et al., 2012) and that individual TF molecules may be engaged in scanning DNA for suitable binding sites and therefore binding at such sites in particular is not functional *per se* (Halford and Marko, 2004).

This last point is consistent with a fourth observation: the number of sites bound by a given TF increases exponentially as occupancy[25] decreases, as predicted thermodynamically from the fact that the number of binding site variants increases exponentially as affinity decreases (Biggin, 2011). In fact, various experimental methods for a number of distinct TFs consistently indicate that, for TFs expressed at a level corresponding to between 10,000-300,000 molecules per cell, >90% will be bound to DNA at any given moment (Biggin, 2011). However, to date it has not been possible to determine what proportion of binding events are functional in terms of gene expression output, or, more importantly, to what extent such variable output regulates biological processes. Remarkably, many studies have taken a dichotomous view of such data, referred to as the "Discrete model, labelling sites above an arbitrary threshold as bound (implying some regulatory role) and all others as not bound, with false positive/negative events considered to arise due to experimental error such as variation between samples. According to such models, TFs typically regulate relatively few target genes. However, any such view appears fundamentally at odds with empirical studies that TF-binding site affinity is a critical regulator of the strength of expression and varies on a continuum. An alternative more in line with the thermodynamic model is the "Quantitative Continuum" model, which posits that for any binding site, TF occupancy increases as a function TF concentration, resulting in a proportionally larger number of regulatory events (Biggin, 2011). Such a model is supported by the facts that overexpression of certain TFs (i.e. increasing expression within a cell in which it is already expressed) is a common cause of cancer (reviewed in Prelich, 2012), and that many TFs exhibit haploinsufficiency (e.g. **Paper III**). Further evidence favoring this model was inferred by analysis of the expression of a broad cohort of genes in the *Drosophila* embryo, in which it was found that only 10% of examined genes were uniformly expressed, whereas approximately 30% were differentially expressed in the embryo by only a 0.1-2-fold difference (Liang and Biggin, 1998). A prediction of the Quantitative Continuum Model is that, within open chromatin, high-affinity sites would tend to be occupied before low-affinity sites.

The ideas that (1) TFs scan DNA for recognition sites, (2) functional binding is concentration-dependent, and (3) that TF binding frequently occurs despite a lack of obvious functionality have led to a "Widespread DNA-Binding" model, in which TFs are viewed as highly expressed and therefore able to bind sites on open/accessible chromatin essentially unaided (Biggin and McGinnis, 1997). The problem with this model is that TFs themselves often exhibit non-uniform expression within a tissue, with some cells exhibiting very low-level TF expression (e.g. **Paper III**). In "Coselective DNA-Binding" models, in which TFs are viewed as too weakly expressed and lacking sufficient sequence-specificity and DNA-binding stability to bind the correct sites without cooperative DNA binding by TF partners (reviewed in Biggin and McGinnis, 1997). Such models have been used to explain the latent specificity of Hox TFs when bound to Pbx proteins discussed above, and imply that TFs scan DNA sequences cooperatively with TF partners. Implicit in Coselective models is the idea that a TF must first correctly bind a TF partner in the three-dimensional space of the nucleoplasm before beginning to scan DNA, but this seems fundamentally at odds with both the purpose of scanning mechanisms (namely to save time searching for the correct binding site) as well as the proportion of TF molecules bound to DNA, given that more time would need to be spent searching for a binding partner, even as most molecules would be scanning DNA without such a partner. Moreover, ChIP-seq analyses indicate that TFs can bind DNA in the absence of any known partners, albeit often at lower occupancy (e.g. Boyer et al., 2005). While this does not exclude a role of protein-protein interactions preceding protein-DNA interactions, these data suggest that a more likely mechanism for cooperativity would entail TF stabilization on DNA upon binding by TF partners. In such a model, extended binding of a site, either of low affinity or at low TF concentrations, would be more depend-

ent on cooperative binding, i.e. dependence on cooperativity is inversely proportional to binding site affinity and/or TF concentration.

Among the early observations most suggestive of the idea that spatiotemporal expression patterns are manifested by direct combinatorial activity of multiple promoter-specific TFs were the striped expression patterns of *eve* and *ftz* in *Drosophila*, which coordinately regulate embryo segmentation and genetically were found to be reciprocally regulated by several TFs (Frasch and Levine, 1987; Harding et al., 1986). Analysis of an element regulating stripe 2 of *eve* identified clustered binding sites for several of TFs whose expression overlapped, and site-directed mutagenesis of each of these motifs resulted in altered expression of stripe 2 corresponding to the activity and expression of the TF for each site, demonstrating how the combinatorial activity of broadly expressed activators and regionally restricted repressors can result in complex and refined gene expression patterns (Stanojevic et al., 1991). More recent genome-wide ChIP studies have found that cooperative TF binding is strongly correlated with the probability of gene expression: in a study of TF binding in *Drosophila* blastoderm embryos, 88% of the 300 most highly bound regions were occupied by eight or more TFs, and these regions were associated with all known targets of these TFs expressed in the blastoderm embryo (MacArthur et al., 2009b).

Cis-Regulatory Module Architecture and Coordination of Gene Regulatory Input

The clustering of TF-binding sites into regulatory islands in the genome, referred to as *cis*-regulatory modules (CRMs), appears to be the *modus operandi* for gene regulation (reviewed in Spitz and Furlong, 2012), and offers an elegant solution to the problem of how to reliably and precisely regulate gene expression, as combinatorial activity effectively increases the length (and therefore specificity) of the regulatory site at a single locus. CRMs are typically characterized by a high degree of sequence conservation across species, and within CRMs, functional TF-binding sites tend to be particularly well-conserved, reflecting the evolutionary constraints on these sequences. Conversely, the strength of conservation of a given non-coding genomic sequence may used in combination with *in silico*-identified candidate binding sites to predict enhancer functionality, as demonstrated for enhancers active in the CNS, in which there is an overrepresentation of Sox-, Pou-, and Homeodomain-class TF-binding sites (Bailey et al., 2006). Despite the apparent power of *in silico* prediction methods, however, efforts to identify CRMs and predict their activity have primarily focused on analyses of genome-wide combinatorial ChIP data (e.g. Zinzen et al., 2009).

CRM architecture, or the arrangement of binding site motifs, is determined by three parameters: binding site order, relative spacing, and relative orientation (reviewed in Spitz and Furlong, 2012). The effects of each on CRM activity depend on whether and how the TFs recognizing each site are able to interact with each other, with cofactors, and with the basal transcriptional machinery. In terms of complementary inputs[26], motif arrangement can direct combinatorial TF activities to regulate gene expression in synergy or additively (or a combination of the two, depending on the number of TFs involved). For example, transcriptional assays in which the CRM for stripe 2 expression of *eve* was used to drive expression of a reporter gene indicated that the TFs *bcd* and *hb* act in synergy at this element (Small et al., 1991). Synergistic activity may be the result of cooperation mechanisms such as those discussed above, including (i) protein-protein interactions between adjacent TFs that stabilize DNA binding; (ii) co-recruitment of additional cofactors; (iii) pioneering by one TF in advance of binding by other partners; and (iv) DNA bending or straightening. Although synergy results in greater sensitivity to a severe reduction of any single input, provided that the concentration of inputs is not limiting, synergistic interactions also buffer gene expression against variation in the concentration of inputs (Masel and Siegal, 2009). This is manifest in switch-like dynamics, which may be particularly advantageous for the regulation of developmentally important genes, e.g. cell fate determinants. By contrast, additive mechanisms result in more graded variation of gene expression, which

may be useful for refinement of processes when target genes encode feedback regulators (Masel and Siegal, 2009).

Perhaps the most intuitive means by which TFs can antagonize each other at a given CRM is competitive DNA binding, in which antagonistic TFs expressed at non-saturating levels share the same binding site, such that competition is resolved when the concentration of one TF becomes meaningfully greater than the other. A well-known example of this is the binding of E-box sites by Hes and neurogenic TFs to regulate processes such as the rate of neurogenesis (Kageyama et al., 2008). A variant of this strategy to circumvent the limited number of TFs that share an identical binding site uses a partially overlapping arrangement between TF-binding sites, as also observed in the *eve* CRM (Small et al., 1991). Synergy and competitive binding are illustrative of the selective constraints on motif positioning, as alterations would likely affect the protein-protein interactions critical to interpret these inputs. A further strategy could rely on variation of TF spacing or differential binding affinity, such that one TF set provides relatively weak input as compared to the other, and the presence of the more strongly binding TFs effectively antagonize the action of the more weakly binding TFs. Such a strategy also offers a means of modulating the level of gene expression.

Although for any given CRM, the sequence conservation implies that there is strong selective pressure on CRM architecture, because of the diversity of TF structures it has been difficult to define generalizable rules of how binding site arrangement regulates gene expression: there's more than one way to skin a cat, as the saying goes. Indeed, comparison of a conserved element across various fruitfly species indicated that although the activity of a CRM can be highly sensitive to alterations in CRM architecture, both the sources and arrangement of inputs can vary significantly across species, implying that when such alterations do occur, they do so in concert with changes in the overall gene regulatory network, as one would anticipate (Swanson et al., 2010).

The modularity of both gene regulatory elements and TFs appears to be an inevitable consequence of evolution, as it offers simple routes to modify specific aspects of TF and CRM function (and consequently gene expression and the gene regulatory network) without destroying others, as binding sites may be modified or added or removed altogether and TF affinity properties altered often by as little as a single point mutation in a given CRM or regions of a gene encoding a TF's DBD. Linear motifs can be added or removed with similarly negligible alterations, and because their interactions are only weak, in most cases this would probably not result in dramatic changes in protein function: indeed, where linear motif function has been analyzed proteome-wide, it does not appear to function in a majority of proteins containing it (Neduva and Russell, 2005). Moreover, what could be called genomic 'microrearrangements', e.g. those caused by viral genome integrations and transposable elements[27], can lead to CRM or protein domain deletion or insertion from other elements at other genomic loci, and DNA replication slippage can lead to duplication of domains or entire genes within a locus (reviewed in Rebollo et al., 2012). At the highest level, genome duplications, of which there have been two in humans ancestors' since the evolution of metazoa, create redundancy on a massive scale, permitting a massive increase in the number of mutations that otherwise could not be tolerated, in turn allowing the gene regulatory network to be sculpted by natural selection and leading to novel functions (de Laat and Duboule, 2013).

GENE REGULATORY NETWORKS

In order to achieve the goal of self-perpetuation, biological systems must satisfy the competing demands that they be (i) efficient in their use of time and resources; (ii) sufficiently robust to be successfully executed under a reasonable range of environmental conditions and a reasonable degree of genetic variation; and (iii) sufficiently sensitive to changes in such conditions to respond in an appropriate manner (Lander, 2011b). The $>2^{20,000}$ potential states of gene expression afforded by the mammalian genome offers many potential solutions to these problems, but it is obvious that the great majority of these states would be non-viable at both the cell and organism levels. Therefore, the essential function of any gene regulatory network (GRN) is to impose constraints on genome-wide gene expression that result in a viable cell and organism. Understanding how this is achieved in transcriptional networks is an ongoing challenge that has been approached at the level of individual TFs, groups of TFs, and at the level of all TFs.

Individually, TF function at the cell, tissue, and organism level has been primarily assessed by genetic studies of loss-of-function mutants, but for decades such analyses were performed in the absence of knowledge about the molecular function of these genes. Following the identification of promoter-specific TFs, molecular cloning methods subsequently made possible gain-of-function approaches, which were used early on to show that misexpression of the TF MyoD in fibroblasts is sufficient to induce transdifferentiation, or reprogramming, of these cells to myoblasts[28] (Tapscott et al., 1988). This experiment demonstrated that TFs can function as critical determinants of cell identity, and was hailed as support for models that each cell type is regulated by a unique master regulator at the top of a hierarchy to direct differentiation of that particular cell identity, reminiscent of the Weismann model of determinants (figure 11; Weismann, 1893). Such a system of regulating cell identity would have the advantage that, once activated, a master regulator could be able to induce an entire program in a short period of time, given that it would, in principle, require relatively few intermediate TFs. However, a consensus has gradually emerged that this is an overly simplistic view, as misexpression of transcriptional determinants in tissues has demonstrated that the ability to reprogram cell identity is context-dependent: for example, the somatic MN determinant Olig2 is sufficient to induce ectopic sMNs only within ventral and intermediate regions of the developing spinal cord, whereas ectopic V2 interneurons are generated at more dorsal levels (Novitch et al., 2001). Such findings are demonstrative of the requirement for cooperative regulation by partner TFs that coordinately determine cell identity, in contrast to models of master regulators directing differentiation in a linear hierarchy.

Global GRN Dynamics

Precisely how TFs regulate these processes at a genomic scale has been greatly aided by genome-wide techniques such as RNA-seq that can be used to compare global gene expression under normal and manipulated conditions, and ChIP-seq. The resulting networks are typically depicted as nodes that represent each gene, and edges (lines) that depict a regulatory interaction, and have, perhaps, been best described as “a hairball” (Lander, 2010). Owing to the relative simplicity of studying unicellular organisms, many of the principles of GRN architecture have been determined in *E. coli* and yeast. For example, mapping of all known interactions between TFs and target genes (including TFs) in yeast found that the number of TFs regulating a given target gene was exponentially distributed, such that ~90% of target genes were regulated by four or fewer TFs (Guelzim et al., 2002). Interestingly, a similar distribution was found for *E. coli*, but with generally fewer TFs per target gene, leading the authors to suggest that this value is dependent on the complexity of the transcriptional machinery, raising the possibility that gene regulation in multicellular organisms might be dependent on the collective activity of larger numbers of TFs (Guelzim et al., 2002). Genome-wide ChIP analyses

of 21 TFs in *Drosophila* are consistent with this idea (MacArthur et al., 2009b). Conversely, however, analysis of the number of genes regulated by a given TF indicated that the distribution was further skewed in a fashion more reminiscent of a power-law, in which the values fit a straight line when *both* the x and y axes are represented on a log scale, the signficance being that a small number of TFs regulate a disproportionately large number of genes — more than would be expected to occur by chance (Guelzim et al., 2002). The former can therefore be thought of as hubs within the GRN, exerting disproportionate influence on network connectivity, with the remaining TFs serving to fine-tune the GRN output (Babu et al., 2004). The idea that some TFs function as hubs offers a compelling explanation for the ability of some TFs to induce transdifferentiation of a comparatively broad range of cell types to a particular fate, whereas others can only do so in more limited contexts, as well as findings that loss of a single TF often does not result in loss of target gene expression (Biggin, 2011). By sequestering most regulatory events to the control a small group of TFs, mutations are more likely to be tolerated, given that mutations occur more-or-less at random in the genome and therefore have a low probability of affecting a network hub (Babu et al., 2004).

In the *E. coli* GRN, the 271 TFs can be grouped into 11 TF families based on the sequence homology (and therefore architecture) of their DBDs, and within each family, the presence and arrangement of other types of domains (e.g. protein-protein interaction domains) is variable, indicating that extensive domain shuffling has occurred (Madan Babu and Teichmann, 2003). Despite these variants, the architecture of each of the 271 TFs corresponds to one of only 74 distinct domain arrangements, indicating that 197 TFs (73%) have arisen exclusively by gene duplication (Madan Babu and Teichmann, 2003). Interestingly, GRN hubs were not affiliated with any particular TF family in *E. coli*, but rather tended to regulate genesets associated with specific cellular activities in a modular fashion (Madan Babu and Teichmann, 2003), implying that hub functionality is not an intrinsic property of any TF class, at least in bacteria. Rather, it raises the possibility that a role as a network hub is an emergent property of the evolutionary elaboration of a given process, and consequently of the functional role of the TF. This offers an attractive explanation of the observation that there are no universal 'stemness' genes governing stem cell behavior, as each stem cell type would have evolved more-or-less independently of the others (Fortunel et al., 2003).

Large-scale analyses of gene expression, in which genes were hierarchically clustered with the genes with which they tend to be most frequently co-expressed, provided unbiased confirmation of this modularity of gene regulation, as genes involved in the same cellular activities, e.g. cell cycle or signaling pathways, had a strong tendency to be co-expressed, such that individual cellular processes tend to be regulated as a unit (Stuart et al., 2003), a phenomenon sometimes called synexpression. This is the great advantage of modular network wiring, as it enables responses to environment cues can be efficiently coordinated by a small group of TFs, increasing adaptability (e.g. Luscombe '04). Consistent with this idea, a substantial proportion of these associations were conserved across eukaryotes (Stuart et al., 2003). Analysis of the component genes in co-expression modules has been used to identify genes associated with specific cell types, e.g. regulators of pluripotency from ES cells and neural stem cell markers from samples of entire brain regions (Mason et al., 2009; Oldham et al., 2008), and this has also served to validate the model. Interestingly, although gene duplication is common, studies in *E. coli* suggest that their corresponding regulatory sequences are rather poorly conserved. Similarly, the significant conservation of gene expression modules implies that they continue to be regulated in a similar manner across species, duplicated TFs show only weak conservation of downstream modules. Together, these data imply that the network is promptly rewired in the course of evolution, both up- and downstream of newly created TFs, suggesting that this could be a key driver of functional diversification (Madan Babu and Teichmann, 2003).

In a seminal study of the entire yeast GRN based on genome-wide gene expression, TF binding, and

known protein-protein interactions (Luscombe et al., 2004), it was found that environmental stimuli as well as the cascades intrinsically determined by the wiring of the GRN both regulate TF expression, altering interactions among TF repertoires. For example, intrinsic programs such as the cell cycle and sporulation were shown to involve broad regulatory activities in which many TFs are expressed but any given TF regulates relatively few genes, a relatively high proportion of which are other TFs. Moreover, TFs could be divided into subsets, one constitutively expressed and others expressed in a temporal relay of contiguously overlapping phases, and it is the interaction between these subsets that appears to propel the GRN through the intrinsic program. Responses to changes in the external environment or other adverse events, e.g. DNA damage, were shown to be characterized by a switch to more focused activities in which a small number of TFs regulate many genes. This architecture appears to be critical to enable rapid responses to acute stimuli. Network hubs were also identified in this study, and although a small proportion of hubs are constitutively expressed, most, like other TFs, are only active under (often multiple) specific conditions. However, those that are expressed under multiple conditions typically do not regulate the same genes in each, implying that combinatorial regulation is both a critical determinant and common feature of global gene regulation in eukaryotes (Luscombe et al., 2004).

GRNs and the Power of Attraction in the Cell State Space

The facts that genes, including TFs, are so often regulated by multiple TFs (all the more so in complex organisms), and that many of these regulators are regulated in turn by their target genes is demonstrative of the non-linear architecture of GRNs. GRNs are not static, but dynamic, reflecting different energy states in which transcriptional activities act as molecular forces that cooperatively or antagonistically try to direct differentiation. These interactions suggest the potential for balance between these forces, evocative of Waddington's description of the epigenetic landscape, in which the lineage trajectory is considered to be regulated by processes that interact and, to some extent, counterbalance each other (Waddington, 1957). Evidence for such an interpretation is particularly strong with respect to the haematopoietic system and pluripotent states, in which individual TFs in the GRN for self-renewal are also critical regulators of alternative fates, e.g. Gata1 and Sfpi1 in common myeloid progenitors, that respectively specify the erythroid and myeloid lineages while mutually antagonizing and cross-repressing each other (Zhang et al., 1999), and Oct4 and Sox2 in ES cells that respectively specify the mesendodermal and ectodermal lineages (Loh and Lim, 2011). Direct evidence for this idea in pluripotent cells was provided by the ability of mesendoderm lineage-specific factors such as Gata3 to substitute for Oct4 in the cocktail of TFs required for somatic reprogramming to the pluripotent state, and abrogation of Sox2-mediated induction of ectoderm lineage specifiers was similarly effective (Shu et al., 2013). It is worth emphasizing that it seems to be the *modus operandi* of GRNs that a single TF can be sufficient to suppress an alternative fate, but there are nevertheless multiple TFs within a network able to suppress a given cell type. In this view, therefore, self-renewal is simply the inevitable consequence of the failure to make a decision, or, as the British Imperialist would call it, "masterly inactivity". The idea that each element of the GRN can contribute to balance the activity of the other elements implies that such balance could stabilize the system sufficiently to be able to sense the surrounding environment and respond appropriately. In such a system, each cell type would correspond to a relatively stable gene regulatory configuration within the larger regulatory landscape, or "state space" of alternative stable states, an idea (borrowed from physics) that was first proposed in the mid-20th century (Delbruck, 1949). In this view, the GRN of an organism is a multistable system.

Stable molecular configurations represent low-energy states to which nearby higher energy states are attracted, and have therefore come to be called "attractors", a concept long equated with cell types by theoretical biologists (Kauffman, 1969; Macarthur et al., 2009a). If the state space is de

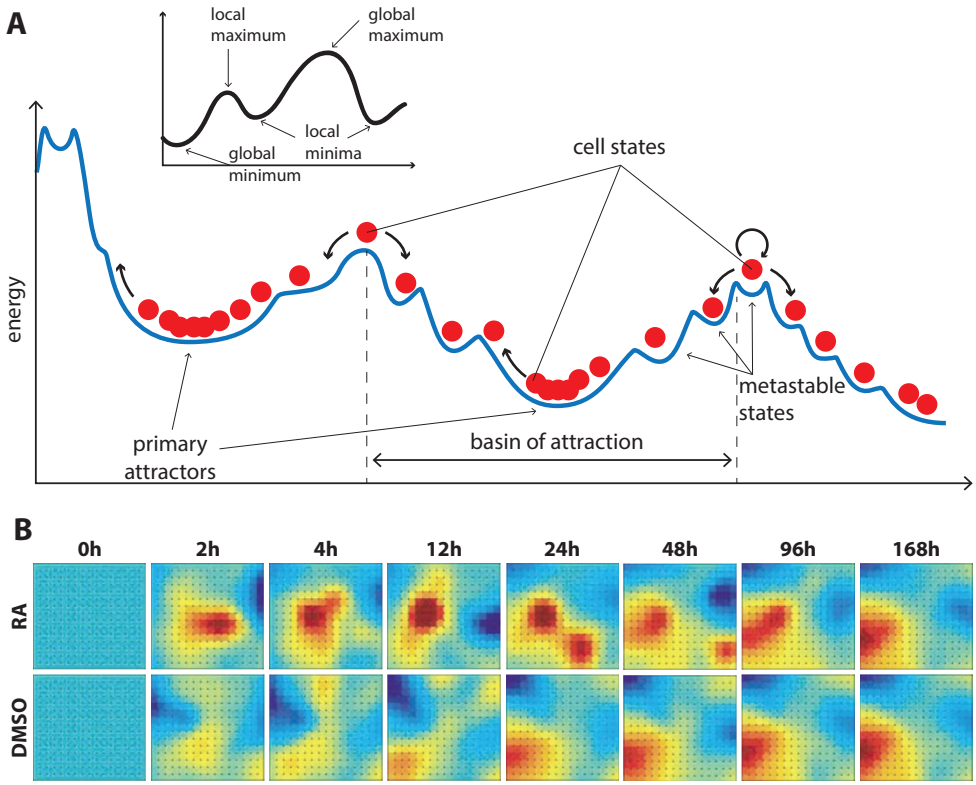

Figure 15. Attractors and differentiation.
(A) Simplified representation of an attractor landscape. Attractors are low-energy (stable) network configurations, or states, toward which the network tends to evolve, like a marble in motion around a bowl that will come to rest at the bottom. However, in dynamic systems, there is no true steady state, so attractors correspond to frequently transited configurations within a basin of attraction. A primary attractor is the lowest energy configuration within a basin, and metastable states to higher energy configurations with their own basins of attraction. When applied to progenitor cell (sub)types, attractors support some degree of self-renewal. INSET: depiction of a mathematical function with terms of description indicated.
(B) Gene expression profiles of promyelocytic cells differentiated to neutrophils by addition of either retinoic acid (RA) or dimethylsulphoxide (DMSO). Plots include only the 2,773 expressed genes that were initially not statistically significantly different between treatments at 0h and that exhibited significant change at some point during the experiment. Modified from Huang et al., 2005.

scribed as a mathematical function of the potential energy of a system, individual cell states correspond to coordinates on that function, attractors to local minima, and the convex region around a local minimum point is referred to as the basin of attraction (figure 15A). When a physical system at equilibrium is perturbed, the transfer of kinetic energy shifts the system away from the equilibrium position; the greater the energy, the further it will be displaced (regardless of whether the stimulus originates intrinsically or extrinsically). If the system is perturbed below a threshold level, the system will eventually revert to the equilibrium position in the absence of further stimulation; however, if the system perturbed beyond a threshold level, it will be displaced to another field of attraction, i.e. it will differentiate. Different perturbations will direct the trajectory of the system in unique ways, and depending on which attractors are nearby, differentiation may be possible to a number of alternative

fates. Alternatively, they could result in differentiation to the same cell type via distinct routes. Viewed through the lens of potential energy, it becomes obvious that state stability will vary quantitatively, rather than qualitatively, such that some states in the vicinity of the primary attractor can also function as attractors, albeit to a more limited extent. Such states are metastable. The presence of these secondary attractors reduces the energy (conceptualized as the height of the basin in figure 15A) that any given stimulus must transfer in order for the system to be (meta)stably displaced from the primary attractor, but also is likely to increase the time the system takes to move into a new basin of attraction because it effectively increases the depth of the basin of attraction. (Compare the height of the entire primary attractor to the total heights of each "sub"-attractor within the basin of attraction in figure 15A)

Experimental evidence for the theory that distinct cell types correspond to attractors in the gene regulatory landscape did not arrive until the advent of transcriptomics, when it was shown that the triggered differentiation of immortalized haematopoeitic progenitors to neutrophils in response to distinct stimuli occurs via distinct trajectories (figure 15B; Huang et al., 2005), proving once and for all that it's not the journey that counts, it's the destination. Using the same experimental system, subsequent analyses showed that although continuous exposure to low-level stimulus can still promote differentiation to neutrophils, premature removal of the stimulus results in a reversion to the initial gene expression state (Huang et al., 2009). Studies of ES cells have demonstrated the oscillating and highly heterogeneous expression of critical transcriptional regulators of pluripotency (Chambers et al., 2007; Toyooka et al., 2008), and shown that inhibition of differentiation signals leads to constitutive ES cell self-renewal, leading to the proposal that the ES cell state is a ground state (Ying et al., 2008), offering indirect support of the wider applicability of the state space model. In studies of common myeloid progenitors in the haematopoietic system, analysis of the expression dynamics of the marker Sca1 and the underlying transcriptome dynamics revealed both a primary attractor state and metastable states, as cell culture of subpopulations fractionated according to the level of Sca1 expression restored the distribution of Sca1 levels: high- and low-Sca1$^+$ fractions gradually reverted to the median, implying the position of the primary attractor, and the medium-Sca1$^+$ fraction re-established the long-tailed distribution with outliers clustering around certain ranges of high and low Sca1 expression (Chang et al., 2008). Importantly, redistribution of Sca1 expression from all compartments occurred under the same culture conditions, implying that gene expression heterogeneity in this system is driven by stochastic mechanisms (Chang et al., 2008).

Causes and Consequences of Stochastic Variation in Gene Expression

"We may regard the present state of the universe as the effect of its past and the cause of its future. An intellect which at a certain moment would know all forces that set nature in motion, and all positions of all items of which nature is composed, if this intellect were also vast enough to submit these data to analysis, it would embrace in a single formula the movements of the greatest bodies of the universe and those of the tiniest atom; for such an intellect nothing would be uncertain and the future just like the past would be present before its eyes."

— Pierre Simon Laplace, 1814 A.D.

"Everything existing in the universe is the fruit of chance."

— Democritus, c. 400 B.C.

Even if Laplace's causal determinism had not been formally disproven by the discovery of quantum mechanics that impart the Universe with a degree of inherent uncertainty, at the relatively more deterministic level of molecules there is a vast body of evidence that physiological processes do not occur identically from one organism to the next. This is due to Brownian motion: the random walks

of molecules in a fluid compounded further by similarly stochastic environmental events. This can result in local fluctuations in molecular abundances that can, in some cases, have disproportionately large effects on the behavior of the system.

Stochasticity can, perhaps, be most readily appreciated by considering plasmids, autonomously replicating DNA molecules that encode extrachromosomal genes conferring traits not on the chromosome e.g. antibiotic resistance enzymes (Paulsson and Ehrenberg, 2001). Many plasmids are maintained at one or two copies in bacterial cells, so cell division could easily result in one daughter cell receiving all copies and the other none. An obvious solution would be to increase the copy number, but at such a low number of molecules, a mere increase from one to two copies doubles the number of molecules and hence the concentration of any encoded gene products. This can also occur following gene duplication on chromosomes; for TF-coding genes, the significance of such events is apparent when considering the Quantitative Continuum model of DNA binding (Biggin, 2011).

It is easy to ignore the role of stochastic behavior in developmental systems: there is comparatively little variation between organisms of the same species, for example, and the development of many organisms is determinate, arguing that chance does not play a functional role in such processes. Moreover, noise in general can be ignored by statistical averaging, and, of course, noise can also result from experimental error. However, even genetically identical organisms developing in homogenous environments are not phenotypically identical, as exemplified by the different ear shapes of identical twins. To be sure, not all intrinsically-determined heterogeneity arises from stochastic transcriptional events: studies in ES cells have demonstrated that mesendoderm markers are enriched during the early G1 phase of the cell cycle, whereas neuroectoderm is enriched during the late G1 phase (Pauklin and Vallier, 2013). Critically, however, eliminating mechanisms of gene silencing increases stochastic behaviors, e.g. spontaneous differentiation of ES cells following mutations of the H3K27 methyltransferase Eed (Boyer et al., 2006). Such experiments indicate that in many circumstances cells go to extraordinary lengths to limit the role of stochasticity in decision-making. The heavy costs are straightforward to quantify when considering single copy plasmids, some of which must produce replication inhibitors at a ratio on the order of 10^4:1 to maintain the correct copy number (Lestas et al., 2010). In determinate lineages, these costs support deterministic mechanisms that mask the underlying transcriptional stochasticity by ensuring asymmetric cell divisions that begin, in *C. elegans*, with the site of sperm entry in the zygote (Gonczy and Rose, 2005).

Definitive demonstrations of stochastic behavior of gene expression had a relatively late start in the molecular era, when it was shown using a synthetic promoter-reporter that the variation of reporter expression levels over the entire cell population was not due to a proportional variation of reporter expression within each cell, but rather to an increase in the probability of any given cell expressing the gene (Ko et al., 1990). Studies in bacteria using two reporters integrated into different genomic loci showed that gene expression between cells as well as between reporters within the same cell is noisy (Elowitz et al., 2002), and analysis of the rates of transcription and translation showed that intracellular noise is due to the former and not the latter (Ozbudak et al., 2002). Experiments designed to visualize the transcription of individual mRNA molecules, in which an mRNA containing a specific recognition sequence would be bound by its binding protein tagged by a fluorescent reporter, demonstrated that transcriptional noise arises from bursts of transcriptional activity interspersed with intervals of inactivity (Affolter et al., 2008).

The example of plasmids is suggestive of the idea that stochastic fluctuations in the rate of transcription would be most likely to impact genes expressed at low levels as compared to those expressed at high levels, due to the likelihood that variation will result in a larger fold-change. However, in general the opposite appears to be the case, in both bacteria and animals (reviewed in Sanchez and Golding, 2013). Importantly, the fact that stochasticity follows a general trend implies that the

primary cause of bursting involves factors that acts at all genes, and given that bursting increases with promoter activity, this suggests that it may be due to kinetics that become rate-limiting as the rate of transcription increases. Recent *in vitro* studies in which 10kb DNA molecules were immobilized and transcribed showed that supercoiling (over-winding and under-winding) of DNA that accompanies transcription results in stalling of the bacterial transcriptional machinery, and that reversal of supercoiling by addition of DNA gyrase, which relaxes supercoiled DNA, resulted in bursts of transcription at the same rate as *in vivo* bursting in *E. coli* (Chong et al., 2014). It is unclear whether the same mechanism contributes to stochastic gene expression in animals, as bursting is characterized by substantially longer intervals than in bacteria, and there are many examples, e.g. housekeeping genes, that do not follow the general trend of increased bursting that accompanies increased gene expression, implying the existence of additional mechanisms (Sanchez and Golding, 2013).

These findings argue that, fundamentally, stochasticity is a problem that must be overcome by biological systems; however, there is substantial evidence that the resultant heterogeneity can be decidedly advantageous, and this is most readily demonstrated in cell culture at low cell density, in which all cells are grown in a largely homogeneous environment. For example, in ES cells cultured under standard conditions for self-renewal, expression of the pluripotency regulator *Nanog* has been shown to oscillate stochastically between ON and OFF states over time, and this has been shown to regulate the probability of differentiation, whereby the presence of *Nanog* efficiently inhibits differentiation and its absence is required but not sufficient for differentiation (Abranches et al., 2014; Chambers et al., 2007). Similarly, $Sca1^{+}$ fractions of common myeloid progenitors are associated with distinct probabilities of commitment to myeloid or erythroid blood lineages, probabilities that are eroded as the distribution of Sca1 expression levels reverted toward equilibrium (Chang et al., 2008). Thus, stochastic regulation of gene expression provides an intrinsic mechanism of cell diversification, creating a functionally heterogeneous population within a genetically homogeneous one for bet hedging (Raj and van Oudenaarden, 2008). GRNs therefore exhibit exploratory behavior (explaining the exploratory behavior of indeterminate lineages): like the crescendos of *A Day in the Life*, their behavior in both determinate and indeterminate development can really be considered aleatoric.

Self-Organized Criticality and Transitions of Cell Identity

"*I say unto you: a man must have chaos yet within him to be able to give birth to a dancing star.*"

— *Thus Spoke Zarathustra*, Nietzche

Stochastically-driven diversification is a form of self-organization: the emergence of a given configuration of the elements in a system from interactions between those elements, rather than as a result of instructions that originate outside the system (reviewed in Halley et al., 2009; Lander, 2011b). Self-organization is only possible in nonlinear dynamic systems because sustained, efficient information transfer is a prerequisite; this requires continual energy input. Of course, efficient information transfer is not sufficient for decision-making: a system in total chaos would have maximal information transfer, but because it is completely disordered, information storage would be impossible. Because both information storage and transfer are required for computation, efficient computation is maximized when storage is possible, but information transfer is rapid, namely high-energy states in the vicinity of a critical point[29]: at the edge of chaos (Halley et al., 2009).

This has led to the suggestion that differentiation may be triggered only once a GRN has approached a critical-like state, becoming extremely sensitive to fluctuations, such that the slightest perturbation could result in a phase transition of the entire system — the straw that broke the camel's back (Halley et al., 2009). In other words, according to this model, stochastically-driven heterogeneity lowers the threshold at which external stimuli can direct the differentiation of selected subpopula

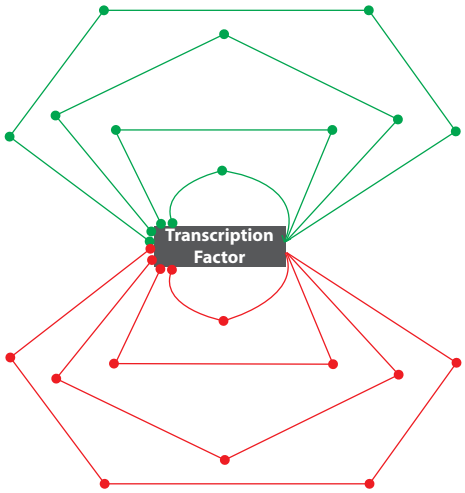

Figure 16. Illustration of criticality in a GRN represented by a transcription factor and the downstream effect of its transcriptional output on its own regulation. Each target gene (the first node at the right of each process) contributes some degree of feedback modulation that may be direct or indirect (intermediate nodes) and ultimately positive (green) or negative (red). The net value of these inputs determines whether the TF remains ON or OFF. Adapted from Halley et al., 2011.

tions, and once the threshold is crossed, the network is suddenly reconfigured, rather like a roller-coaster that, having slowly pushed and pulled its way to the top, descends precipitously to the bottom on the other side. The classic illustration from the physical world is of sandpiles to which single grains of sand are added: when the pile reaches the critical point, a single grain could trigger an avalanche, or might not, depending on *exactly* where and how it falls (Bak et al., 1987). This is consistent with findings that cell types correspond to attractors with intermediate metastable states that are differentially predisposed to differentiate along distinct lineage trajectories (Chang et al., 2008). Likewise, mathematical simulations of gene regulatory circuits indicate that stochastic variation in the intensity of a sustained perturbation can suffice to cross a critical inductive threshold once the system has reached a baseline steady state just below that threshold (e.g. Lai et al., 2004). Consider the expression of a TF closely associated with the identity of a given cell type (figure 16): in that cell type, some (if not all) of its target genes are likely to positively or negatively regulate its expression to some extent, either directly or via a certain number of intermediate regulators. The balance in the strength of positive and negative inputs determine whether it will remain expressed (a zero-sum game), but if the strength of each is approximately equal, stochastic events could lead it to be switched off (Halley et al., 2012). Over time, the output branches that feed back to the TF will vary in activity (as longer branches take more time to take effect), altering the relative strength of inputs. Viewed in the context of the broader cell state, and given that a given TF expressed in a progenitor typically will eventually be switched off, one could infer that the GRN can drive the system toward criticality, and therefore the 'edge of chaos' would correspond to an attractor, albeit a metastable one. (Similarly, in the context of a mutation of one of these genes, the conditions under which the strength would be approximately equal would shift to favor one or the other expression state and/or cell fate.)

Of course, to understand whether such a model applies to actual GRNs will firstly require determination of whether transitions between attractors (or at least primary attractors) should be considered differentiation events or only a subset, and if the latter, where one draws the line. For example, it is unclear whether the subdivision of progenitor pools e.g. in the developing nervous system, should be considered differentiation or merely diversification, or to what extent it is simply semantics. Likewise, the gene expression oscillations and the reversibility of commitment described above that are consistent with metastable intermediate states suggest that differentiated states emerge from gradual, multilayered diversification steps. It therefore seems possible, if not probable, that such minor transitions would not

correspond to “avalanches” Moreover, perturbations that are sufficiently large would presumably enable the network to bypass certain metastable states and criticality, much as a strong gust of wind can trigger an avalanche. To draw on the “edge of chaos” concept, it may be that criticality is only required when extremely precise interpretation of environmental events is required to distinguish between competing, finely balanced instructions (Halley et al., 2012).

Motifs in GRN Circuits

Global analyses of TF connectivity have determined that GRN circuitry consists of patterns of TF connectivity, or motifs, that occur in the GRN more frequently than expected by chance, suggesting that they could provide a selective advantage through characteristic regulation of information processing in transcriptional circuits (reviewed in Alon, 2007, from which the following examples are drawn unless otherwise stated).

In the simplest possible regulatory event (figure 17A), a TF, *T*, activates expression of a target gene, *t*, once its concentration reaches a *t*-inducing threshold, which is governed by the affinity of the binding site for *T* in the regulatory region of *t*. Once induced, the rate of target gene transcription and protein synthesis will result in a buildup of its protein product, whereas when the TF falls below an inducing threshold, the gene will be repressed and its protein product will exponentially decay with a half-life dependent on (i) its inherent stability; (ii) whether the functional protein is actively degraded; and (iii) the dilution effect of cell division. The rate of production and degradation will determine the steady state level (V_{MAX} in kinetic terms) of expression and hence the response time (0.5 x V_{MAX}). If *t* is also a TF that binds its own promoter, it may influence its rate of production once it reaches a critical concentration threshold, resulting in positive or negative autoregulatory feedback, depending on whether it acts as an activator or repressor, respectively. The significance of these autoregulatory loops lies in their opposing effects on the response time as compared to simple regulation designed to reach the same steady state level: negative autoregulation reduces the response time, whereas positive autoregulation increases it (figure 17A). However, to achieve the same steady-state the requires an increased or decreased rate of production for negative and positive feedback, respectively, or increased or decreased rate of degradation for positive and negative feedback, respectively. Provided that it is sufficiently strong, positive autoregulation of the downstream TF can result in hysteresis, or memory, after the upstream TF has been downregulated or switched off.

A second family based, in its purest form, on the model of simple regulation is the single-input motif (SIM), in which *T*, acting alone, activates multiple target genes. This motif can be used to induce a battery of target genes in a temporally defined manner, when target genes are regulated by differential *T*-binding sites (figure 17B).

A third category of motifs is the feedforward loop (FFL), which is comprised of three TFs, *X*, *Y*, and *Z*, in which *X* regulates both *Y* and *Z* and *Y* also regulates *Z*. Of the eight possible variants, at least four can be readily identified in the GRN studied in **papers I-IV** (see also figure 17C). There are four coherent and four incoherent FFLs in which *X* directly regulates *Z* in the same manner as it indirectly regulates it via *Y*, or in which the direct regulation of *Z* by *X* is the opposite of its indirect regulation of *Z* via *Y*, respectively. Each FFL has unique dynamic properties. For example, in type 1 incoherent FFLs, *X* activates both *Y* and *Z*, whereas *Y* represses *Z*, resulting in a pulse of *Z* expression before *Y* reaches a *Z*-repressing threshold. In addition to pulsing, it can also detect fold-changes of TF expression that are independent of the baseline level (figure 17C; Goentoro et al., 2009). A key distinguishing quality of each FFL is its contribution to noise: FFLs in which X represses *Y* have been shown to be noisy when at their steady state ON level, in contrast to those in which X activates *Y*, which result in noise only when in the OFF state for an extended period (Kittisopikul and Suel, 2010).

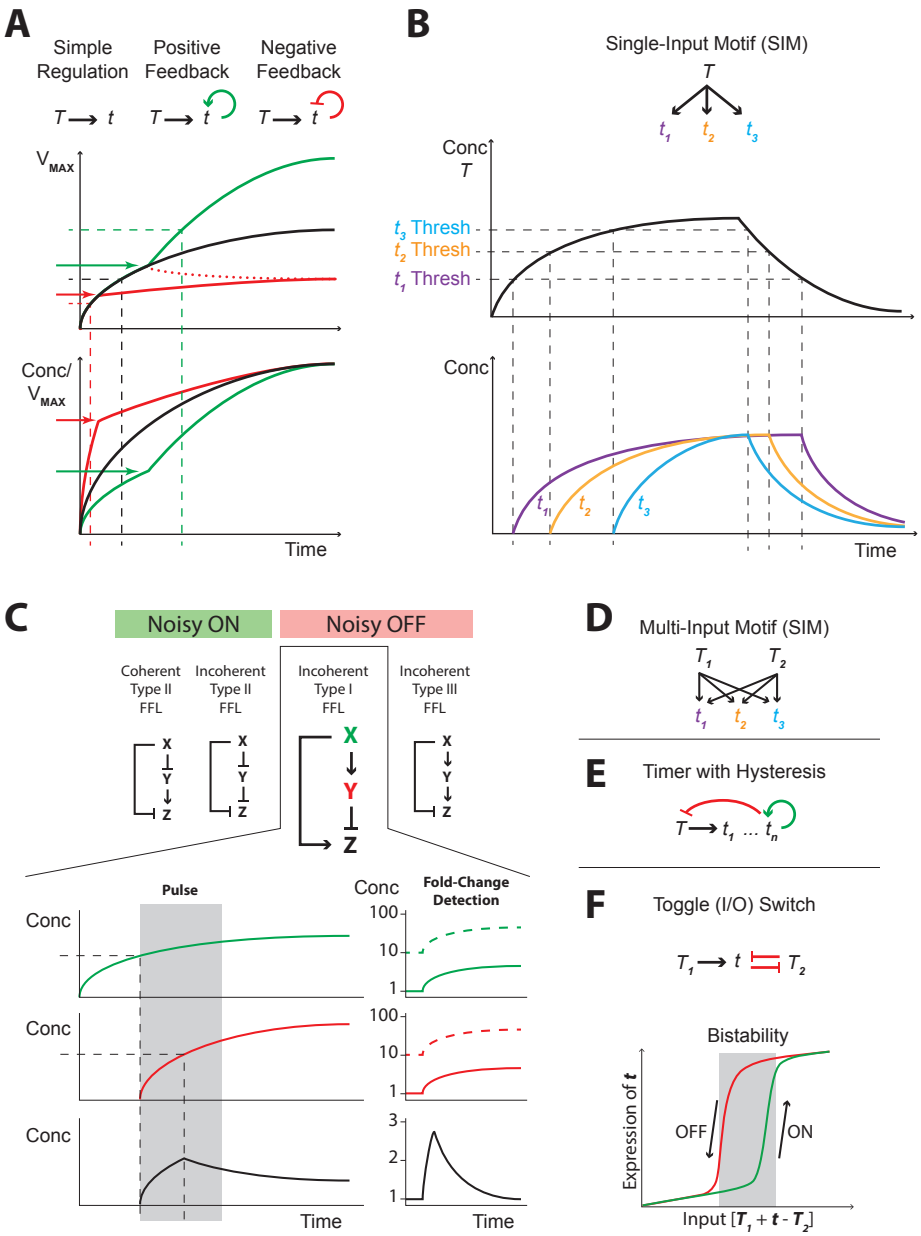

Figure 17. GRN motifs have unique dynamic properties.
(A) Illustration of the effects of feedback on a simple motif. TOP: motif structures. MIDDLE: Graph depicting target gene expression dynamics for each motif relative to V_{MAX} for that motif, illustrating that autoregulation affects the steady state level of expression. Red dotted line illustrates how expression profile changes with higher threshold for negative autoregulation, resulting in a pulse. BOTTOM: values from upper chart plotted as conc/V_{MAX} to illustrate how response times are affected by autoregulation. Dashed lines indicate response time (0.5 x V_{MAX}) of each motif, arrows indicate threshold level of input to initiate autoregulation. Simple regulation (black); positive autoregulation (green); negative autoregulation (red).
(*Continued on page 44*).

This is due to the random binding and unbinding events of *X* and *Y* to the promoter of *Z* that regulate the amount of time *Z* is in the high-, medium-, and low-expression states. Analysis of the functional pathways regulated by FFLs in *E. coli* identified two classes preferentialy regulated by each subset of FFLs: noisy ON state FFLs tend to regulate processes in high demand, whereas noisy OFF states typically regulate more rarely needed responses. This stochastic behavior therefore seems to provide opportunities for exploration of alternative network states that may increase adaptability (Kittisopikul and Suel, 2010).

A caveat in the study of motif dynamics stems from the fact that individual motifs typically overlap with or operate immediately up- or downstream of other motifs in the GRN circuitry, and in such cases the behavior of the upstream motif is influenced by the downstream motif (Knabe et al., 2008). For example, if the TF contributing to both the up- and downstream motifs is autoregulated, this connectivity slows the response time of the upstream circuit, as binding to target genes can protect it from being degraded (Chalancon et al., 2012). Thus, the presence of a given motif is not always indicative of a single stereotyped behavior.

There are numerous variants of these simple motifs, some of the architecture and functions of which are described as follows. Indirect autoregulation is a variant of this category of motifs in which the downstream TF loops back to repress the upstream TF, creating a timer whose interval increases with the number of intermediate TFs or as TF binding site affinity decreases (figure 17E). A simple oscillator can also be engineered if it is the inducing TF that is positively autoregulated rather than the downstream TF (Ninfa and Mayo, 2004). Negative regulation may also be employed to generate a double-negative motif in which two target TFs mutually repress each other, acting as a toggle (I/O) switch that results in bistability, i.e. a system with two mutually exclusive states that are both stable under some of the same conditions (figure 17F). A variant of the single-input motif is the multi-input motif (MIM), in which multiple TFs activate the same set of target genes (figure 17D; Babu et al., 2004), resulting in refinement of gene expression. However, this can be further subdivided according to the logic of the motif: if it is AND-gated, the TFs activate target genes in synergy, whereas OR-gated motifs are characterized by some degree of redundancy, as is typical of more recently duplicated TFs that bind the same recognition sequence, and the inputs are additive if both regulators are limiting. MIMs become increasingly common and pure SIMs increasingly uncommon as a function of organism complexity.

Hierarchical Organization of GRN Sub-Circuit Outputs

Overlapping motifs form larger sub-circuits with distinct topologies, and just as motifs have characteristic dynamic properties, molecular dissection of a number of developmental GRNs suggests that specific developmental activities are mediated by corresponding sub-circuits across populations of

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(*Cont'd from page 44*)
(B) For a single input motif (TOP), target gene expression dynamics (BOTTOM) depend on TF-binding site affinity, such that higher concentrations of input TF (MIDDLE) are required to activate a target gene with a low-affinity site.
(C) The four (of eight possible) feedforward loops identifiable in the GRN studied in papers I-IV. Two at left exhibit noise when Z is being turned ON; the two at right exhibit noise when Z is being turned OFF. The dynamics of the type I FFL is shown in a scenario in which baseline expression of X = 0 (left) or 1 (right).
(D-F) Variants of motifs in (A) and (B).
Adapted from Alon, 2007.

progenitors within the embryo (Davidson, 2010). These sub-circuits must accomplish the related functions of imparting cell identity and performing the cell's role in shaping the embryo, which is achieved in a stereotypical flow of GRN sub-circuit outputs that successively specify and compartmentalize progenitor pools in response to environmental and/or intrinsically generated cues, dynamically locking in new states through hysteresis, and excluding alternative fates (figure 18A). The exclusion of alternative fates is, of course, part of the process of establishing and maintaining a cell state and therefore could be considered an aspect of "dynamic state lockdown", but in terms of sub-circuits it is often distinct, being carried out by dedicated repressors, whereas the latter involves feedback maintenance of the lineage specifiers by transcriptional activators. Importantly, any given TF may be involved in multiple steps of the flow: one of the clearest examples based purely on expression is Sox2, which is expressed continuously within the neural lineage from the zygote until postmitotic differentiation. It has been proposed that only a small number of sub-circuit types regulate all developmental processes (Davidson, 2010), with distinct sets of regulators comprising each sub-circuit of the same type, all of which would have arisen by convergent evolution, like GRN motifs.

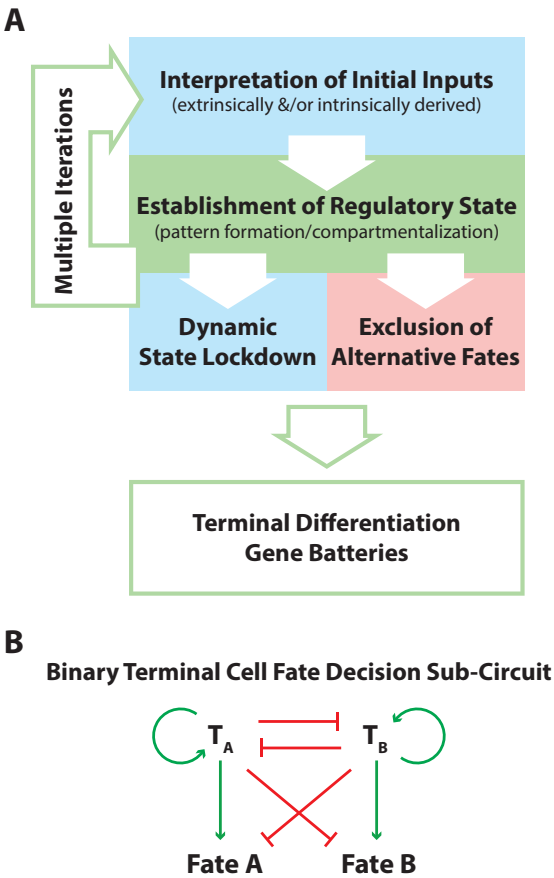

Figure 18. GRN sub-circuit flow.
(A) Flow chart depicting the functions of GRN sub-circuits required to execute the developmental program. A TF within a given sub-circuit may also play roles in other sub-circuits. For any given cell fate, the network cycles through multiple iterations of the flow, in each of which different complements of TFs are used to interpret environmental signals and compartmentalize populations of cells, the identity of which must be self-sustaining and exclusive of alternatives. Eventually, this terminates in the activation of differentiation gene batteries that promote cell cycle exit and terminal differentiation.
(B) A sub-circuit characteristic of the stem cell/progenitor pools of the haematopoeitic system that incorporates many of the functions of each type of subcircuit: two co-expressed TFs, induced by extrinsic factors, each maintain their own expression and promote a differentiation program for a given fate while repressing each other's expression and the alternative fate. Once one reaches a critical threshold fold-difference over the other, differentiation is triggered and the alternative fate fully suppressed. The prevalence of this particular sub-circuit architecture in cell fate determination is controversial (see text).

The prevalence of certain sub-circuits is controversial, particularly those relating to the exclusion of alternative fates (e.g. Davidson, 2010; Zhou and Huang, 2011). While it is commonly accepted that active transcriptional repression of nearby alternative states in the state space (e.g. the undifferentiated

parental cell state of a differentiated cell, as well as the alternative states to which the parental cell was competent to differentiate) is a general feature of the regulatory architecture governing each cell type, studies of the haematopoetic system have led to the proposal that the branch points of all lineages and sublineages are ternary, i.e. that a parental cell type can either self-renew or differentiate into one of two alternative fates that are each distinct from the parental state (Enver et al., 2009; Zhou and Huang, 2011). Moreover, with the support of cis-regulatory analyses of the GRN governing cell fate determination in the haematopoeitic system, it has been suggested that these branch points are universally regulated by a sub-circuit in which each of the two bifunctional TFs of a double-negative motif positively autoregulate themselves, providing sufficient network stability to enable self-renewal in the absence of signals to differentiate. Moreover, each acts as the input of a single input motif comprising a differentiation program, as well as a repressive single-input motif, such that each suppresses many of the other's downstream target genes (figure 18B; Enver et al., 2009; Zhou and Huang, 2011). One of the problems with this is obvious: not all stem cells are bipotent, e.g. sperm stem cells. In other cases, e.g. the branch point for trophectoderm and ICM differentiation in the early embryo, it has been pointed out that the two TFs required for each fate, Cdx2 and Oct4 in this case, respectively, are co-expressed for an extended time and initially not antagonistic. It has therefore been argued that this type of sub-circuit operates only during terminal binary cell fate decisions (Davidson, 2010). However, given that these TFs eventually become antagonistic, the activation of an additional TF(s) or cofactor(s) must alter their functionality, raising the possibility that such a sub-circuit could operate at this branch point. Another issue is that this particular sub-circuit accomplishes multiple distinct tasks of the GRN output flow (dynamic state lockdown, exclusion of alternative fates, and priming of differentiation drivers) that require activator and repressor functions. However, not all fate-determining TFs are bifunctional: dedicated repressor TFs may also depend on activators to coordinately regulate cell fate determination, as is the case in the terminal binary cell fate decision GRN sub-circuit studied in **papers I-IV**. Therefore, at least in some cases, there are multiple sub-circuit structures capable of performing precisely the same type of task. Moreover, it is notable that many of the sub-circuits described by Davidson (2010) that regulate different processes are fundamentally analogous and/or overlapping in terms of wiring, such that no fewer than eight (of thirteen) have been shown to be integral to the function of the repressive GRN regulating neuronal subtype selection studied in **papers I-IV**. It may therefore be that the deployment of functional categories of developmental sub-circuit is less varied over the course of development but the precise sub-circuit architectures themselves more varied than the prevailing wisdom suggests.

Extrinsic Regulation of GRNs

A GRN that cannot respond to changes in the environment would be like a brain without the senses. The generation of ectopic structures following grafting experiments, e.g. limb field transplantation (Harrison, 1918), proved that signaling amongst progenitors plays a critical role in development. Complementing this, newt-to-tadpole or tadpole-to-newt transplantation of flank ectoderm to the future oral area resulted in formation of a mouth, indicating that the same signals direct ectodermal fates across species, but, strikingly, the mouth formed was that of the donor species rather than the host, demonstrating a genetic specificity of induction in which signals direct the expression of whatever genes are available to them (Spemann and Schotte, 1932). It is conceivable that such signals could be biochemical or mechanical (and, of course, examples of each are now well documented), but whereas mechanical signaling must be contact-mediated, biochemical interactions between cells could employ either juxtacrine (interactions between adjacent cells via cell membrane-tethered molecules) or paracrine (secreted, diffusible molecules that could act at non-adjacent cells) signals. For unicellular organisms living in aqueous environments, signaling by untethered molecules, e.g. in

nutrient sensing, obviously must predominate in GRN regulation, but it is feasible that a multicellular organism could develop exclusively using juxtacrine and/or mechanical signaling, allowing direct control over which cells receive a given signal. Proof of a role for paracrine signaling in development was provided by studies of kidney tubule development, in which a filter, used as a physical barrier between inductive spinal cord and responsive metanephric tissue, was unable to prevent tubule formation (Grobstein, 1956).

Genetic analyses have since revealed that most tissue patterning and morphogenesis is regulated by a mere seven families of pleiotropic signaling pathways (Gerhart, 1999): hedgehog (Hh, including sonic hedgehog, the primary focus of this thesis), transforming growth factor-ß (Tgfß), nuclear receptor, wingless (Wnt), Notch, receptor tyrosine kinase, and Jak-Stat signaling (figure 19), of which only the Notch pathway is juxtacrine. This underscores the importance of paracrine signaling in regulating cell identity, while ruling out any preconceived notions of dedicated signaling master regulators, but also raises the question of how these secreted signals can elicit robust, spatiotemporally precise GRN responses that limit inappropriate events in their absence.

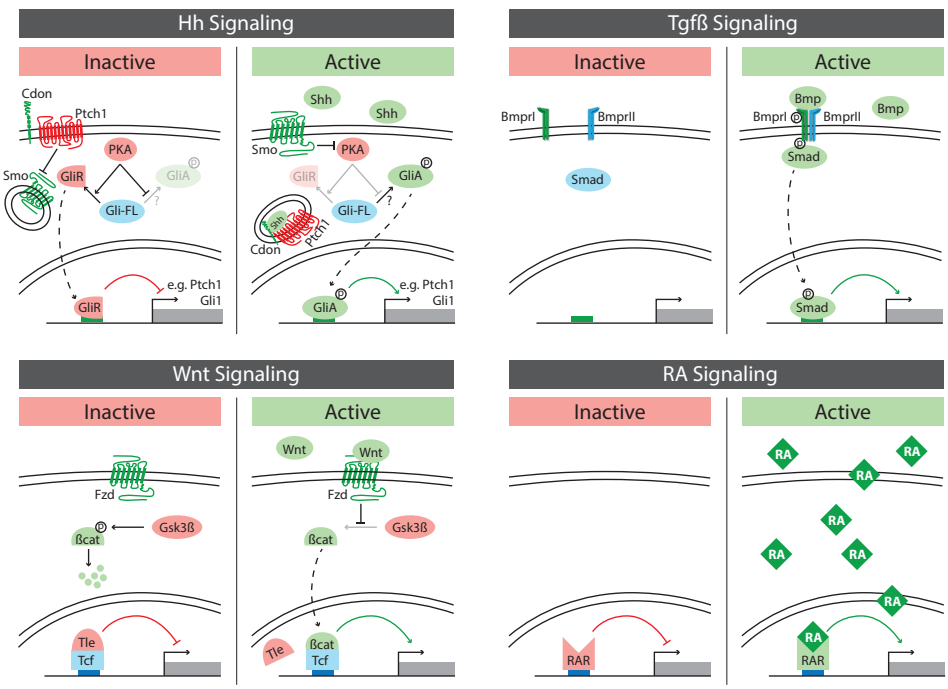

Figure 19. Schematic summaries of the basic components of the four pleiotropic signaling pathway families of direct relevance to this thesis, in the complete absence or presence of high signal concentrations.

Outside-In: Signal Reception

In order to regulate the GRN, a molecular signal originating outside the cell must somehow lead to modification of the activity of one or more TFs inside the cell as a direct result of exposure to the signal. A simple solution would be to use membrane-permeable ligands that diffuse directly to the nucleus where it binds a TF doubling as its own nuclear receptor, e.g. retinoic acid (RA) binding of

its own receptor, RAR (figure 19; Gavalas, 2002). Most signals, however, are proteins with an electrical charge and therefore not membrane permeable (Alberts, 2015). Such signals therefore require receptors at the cell surface that act as an interface between the extra- and intracellular environments. These receptors must be membrane-spanning, such that binding of the ligand to the extracellular domain leads to a conformational change in the intracellular domain that could alter the activity of the signal-regulated TF via a few distinct mechanisms, including (i) releasing it from the receptor itself to allow translocation to the nucleus, as occurs in the Notch pathway (Shaya and Sprinzak, 2011), (ii) transducing the signal by regulating post-translational modification of the TF, e.g. by enzyme-dependent phosphorylation of R-Smads in Bmp signaling of the Tgfß family (figure 19; Le Dreau and Marti, 2013), or (iii) by regulating post-translational modification of a cofactor, e.g. inhibition of default proteolysis of ß-catenin, a cofactor of TFs of the Tcf family that mediate Wnt signaling (figure 19). The Hh pathway is unusual in this respect, as its receptor, Patched (Ptc), has been shown genetically to be a repressor of the pathway, inhibiting the constitutive function of another receptor, Smoothened (Smo), which regulates the post-translational processing of members of the Gli family (Gli1-3) of TFs to either Gli activators (GliA) or, in the case of Gli2 and Gli3, to Gli repressors (GliR; figure 19; Alcedo et al., 1996; Chen and Struhl, 1996; Sasaki et al., 1999).

Three Habits of Highly Effective Signaling

Both the competence to respond and the type of response (i.e. transcriptional activation or repression) could be regulated to result in distinct outcomes of signaling. The former could be accomplished simply by repressing the expression of essential components of the signaling pathway, but the latter requires a more elaborate scheme, dubbed the "three habits of highly effective signaling pathways" (Barolo and Posakony, 2002), in which (1) a variety of partner TFs not otherwise associated with the signaling pathway provide specificity in target gene selection, by virtue of their differential expression across the field of responding cells; (2) signal-regulated and partner TFs act in synergy, preventing low-level expression when the target gene is supposed to be off; and (3) the default expression state of target genes is the opposite of the state induced by signaling. Thus, exposure to ligand results in a switch between active and repressed states. Signal-regulated bifunctional TFs offer the simplest way to execute such a switch, a strategy used by the Wnt pathway with Tcfs, RA signaling with RARs, and the sonic hedgehog (Shh) pathway with Gli2 and Gli3 (figure 19; Sasaki et al., 1999). The three criteria for effective signaling were initially framed in terms of signal-mediated transcriptional activation and default repression, a feature of all major developmental signaling pathways (Barolo and Posakony, 2002). This is all very well for bifunctional TFs; complications begin to arise, however, when activator and repressor functions are mediated by distinct TFs. For example, default repression of targets of signaling by Dpp, the *Drosophila* homolog of Bmp, is mediated not by the Smad homolog Mad, but by Brinker (Brk), a TF whose binding site partially overlaps that of Mad. As Dpp signaling swells, *Brk* is directly repressed by Mad via a Brk-insensitve site, leading to derepression of Dpp target genes. In other words, *Brk*, the Dpp default repressor, is therefore active by default, despite being directly regulated by Mad, which hitherto was considered exclusively an activator (Pyrowolakis et al., 2004). A substantial and growing body of data indicates that many target genes of most, if not all, major signaling pathways are activated by default and repressed by signaling (Affolter et al., 2008), including some using bifunctional TFs. In *Drosophila*, it has been shown that the default activity of Pangolin, the Tcf homolog, is dependent on the DNA-binding site: a short, canonical site results in the usual default repressor activity, whereas the additional binding of another region of Pangolin/Tcf to a 'helper' site results in a conformational change in Tcf and hence default activation of target genes, and also inverts the function of ß-catenin (Zhang et al., 2014). A number of target genes of Shh signaling have also been identified that are regulated inversely to the default

repression model, including three Shh-binding receptors, *Cdon*, *Boc*, and *Gas1*, that assist in the loading of Shh to Ptch1 (figure 19; Allen et al., 2007; Tenzen et al., 2006). Whether Gli proteins mediate this activity of Shh is not yet known, although the upregulation of *Cdon* expression in *Gli3*$^{-/-}$ mutant mice is consistent with this possibility (McGlinn et al., 2005).

Cross-talk Between Signaling Pathways

With all the signaling going on in a tissue, a readily available means of further refining the output of a pathway is through cross-talk between signaling pathways, which could occur at any point in the pathway, in principle: from production of the signal to target gene regulation, and could be (i) direct, (ii) sequential, or (iii) cooperative. (i) Direct cross-talk refers to the co-selection and/or sequestration of shared components. In Shh signaling, for example, genetic ablation of protein kinase A (PKA) function results in constitutive activation of the Shh pathway, indicating that it acts as a critical negative regulator[30] (Tuson et al., 2011), and biochemical analyses have shown that this activity involves phosphorylation of full-length Gli TFs at multiple sites, inhibiting GliA formation and triggering GliR formation (figure 19; Niewiadomski et al., 2014; Pan et al., 2006; Wang et al., 2000). PKA is also a critical regulator of the Creb pathway, in which it is activated by cyclic adenosine monophosphate (cAMP) and in turn phosphorylates Creb (Kotani, 2012). Several studies have provided evidence that the G protein-coupled receptors, Gpr161 and Adcyap1r1 (Pac1R), increase cAMP levels and thereby negatively regulate the Shh pathway in the developing CNS in a PKA-dependent manner (Cohen et al., 2010; Hirose et al., 2011; Mukhopadhyay et al., 2013; Niewiadomski et al., 2013). (ii) In sequential cross-talk, the target gene(s) of one pathway are critical components of another. During neural development, for example, ß-catenin/Tcf7l2-mediated Wnt signaling has been shown to upregulate *Gli3* expression, thereby increasing GliR to counteract Shh signaling (Alvarez-Medina et al., 2008). Cooperative cross-talk refers to regulation of the same target genes. For example, Shh and RA have been shown to regulate some of the same target genes during neural patterning (Novitch et al., 2003). Although such studies suggest mechanisms in which gene expression can be refined or restricted, they do not offer an overarching logic of tissue-specific gene expression (see below and **paper IV**).

Response Signal-arity

If stem cell competence is binary or multinary (figure 10), this must be reflected in the GRN architecture with respect to signal inputs. On the face of it, binary responsiveness is the simplest to structure, requiring a signal only for the correct timing of a possible response, and indeed is only capable of interpreting it as an all-or-none switch. The classical example of this is the newt-tadpole ectoderm transplantation (Spemann and Schotte, 1932). In other words, binary responses act as gates, reflecting the poised state of a network that has already determined how it would execute a process, if the situation allows. In cases of multinary responsiveness, signals act instructively, producing a range of (≥3) possible interpretations, i.e. no response or one of two or more alternative responses, such that the quality of the signal must be gauged by the responding cell. Being molecular, the quality of a signal could be affected by modifying the signal to have differential receptor affinity, different transport modes (and hence kinetics), or possibly resulting in differential modification of the conformation of the receptor. Shh, for example, can be modified by addition of a variety of lipids, which influence ligand potency and diffusivity (Long et al., 2015).

Ligand Concentration-Dependent Responsiveness

A generally better understood (albeit related) mechanism, however, is to vary the availability of signaling pathway components, the most effective being those regulating the rate-limiting steps: foremost among these are the rate of ligand production, release, diffusion, and/or degradation, as these

parameters determine the ligand concentration at a responding cell. An obvious way to interpret a variable extracellular concentration of ligand would be to produce a correspondingly variable concentration of activated signal-regulated TF. The elegance of such a readout mechanism becomes evident if that TF is the input of a single-input motif in the GRN (figure 17), such that distinct target genes are activated by distinct concentration thresholds of ligand exposure.[31]

A key question, then, is how the ligand concentration is measured. One way or another, this must be a function of receptor occupancy, i.e. the number of bound receptors and average duration of binding. For a simple pathway, i.e. a receptor that directly activates the pathway, the absolute receptor occupancy will determine the level of pathway activation, as increasing or decreasing the total number of receptors will not affect the transduction flow downstream of the receptors that are already occupied, provided that a minimum number of receptors are actually present for that activation. Such an absolute occupancy model has been invoked to explain the output of activin signaling during mesendoderm development (Dyson and Gurdon, 1998). That being said, increasing the number of receptors could still increase the pathway activation by increasing the probability of ligand binding, and, indeed, activin receptor occupancy was found to be very low, even during high-level responses (Dyson and Gurdon, 1998). However, if the receptor is a negative regulator, as genetic studies indicate that Ptc is of Smo, then ligand binding would lead not to direct activation, but to derepression, such that the number of *un*bound receptors becomes the critical variable, and increasing the number of repressors would silence the pathway. Consequently, positive feedback upregulation of an inhibitory receptor could be a useful means of terminating signaling, provided that it is responsive only to high levels of signaling (whereas a high sensitivity would be incompatible with absolute models due to premature termination of signaling).

The discovery that *Ptc* is a direct target of the Hh pathway, upregulated in response even to low concentrations of Hh (Marigo and Tabin, 1996), therefore argued against an absolute occupancy model in Hh signaling, implying, rather, that Ptc is bifunctional: repressing the pathway when unliganded but activating it when occupied, such that responding cells would interpret the ratio of unoccupied and occupied receptors. By contrast, for an absolute model, the only effect would be to reduce the ligand available to other cells, in which case a cheaper strategy would simply be to reduce the amount of ligand. This was demonstrated by an elegant set of experiments with a Hh-insensitive form of Ptch1 ($Ptc^{\Delta loop2}$) that constitutively inhibits Smo, but was found to have a comparatively mild inhibitory effect on Shh signaling in *Drosophila* cells that *already* expressed Patched, whereas in *Patched* mutants, the same level of $Ptc^{\Delta loop2}$ was able to completely suppress ectopic expression of Shh target genes. Moreover, through experiments in which Hh was translationally fused to Patched to create a constitutively active receptor, when co-transfected with $Ptc^{\Delta loop2}$, it was shown that only a ratio $\geq$ 3:1 was sufficient to activate Shh signaling (Casali and Struhl, 2004).

The mechanism of receptor-mediated inhibition may add another dimension: whereas if, for instance, the inhibitory receptor binds and sequesters the activator or an interacting partner to form a stable, inactive complex, the relationship between the two receptors would be stoichiometric; on the other hand, if the inhibitor acts catalytically, a much higher level of ligand occupancy would be required to relieve activator inhibition, as observed in hedgehog signaling, in which a 50-fold excess of Smo over Patched was required to achieve half-maximal pathway activation (Taipale et al., 2002). Subsequent studies have provided evidence that Patched, which bears structural and sequence homology to a class of bacterial lipid and toxin transporters, transports sterols that signal to Smo, inhibiting it in the case of provitamin D3 and activating it in the case of oxysterols, although the identity of the ligands that normally mediate signaling has yet to be determined, but cholesterol depletion inhibits Smo (reviewed in Mukhopadhyay and Rohatgi, 2014).

Concentration-dependent signaling typically involves high affinity of receptors for ligands, so if

the bound receptor is not degraded, the slow dissociation rate will result in a maintenance of signaling long after the source of the signal has ceased production, resulting in an averaging of noise in ligand production over time, as well as slow termination of signaling (the "ratchet" effect; Dyson and Gurdon, 1998) — even if accompanied by inhibitory receptor upregulation, although this would partially offset these effects. However, in response to Hh signaling, ligand-bound Ptc is internalized and degraded, ruling out such a mechanism in this pathway (Incardona et al., 2002).

As evolution is blind to the future, mechanisms can take shape that are costly as compared to alternative systems, e.g. the >10-fold higher occupancy observed for Hh as compared to activin in response to high concentrations of signal (Casali and Struhl, 2004; Dyson and Gurdon, 1998), and presumably increased receptor production and destruction. Nevertheless a number of potential advantages of the complexity Hh reception apparatus can be readily identified. (1) Default repression of pathway could provide tighter regulation of pathway activity. (2) The bifunctional catalytic activity of Ptc may lead to spatial averaging of noise by activating nearby cells via sterol secretion, and (3) also demands relatively low-level expression of inhibitory receptor for efficient inhibition, offsetting the requirement of higher receptor occupancy. (4) Receptor internalization and degradation upon ligand binding reduces the diffusivity of the signal, so fewer cells are exposed to high ligand concentrations, (5) It also reduces noise resulting from variation in ligand production, and (6) results in faster termination of signaling once the signal is withdrawn. (7) Receptor upregulation similarly reduces diffusivity and (8) shortens the response time through negative feedback.

Transduction Flow and Temporal Adaptation

An important principle of signaling pathways is that there is a continuous transduction flow, i.e. TF post-translational modification, over the time the receptor is bound by ligand, and the rate of flow is therefore constant for a given value of receptor occupancy, assuming there is no feedback regulation of other downstream components (Freeman and Gurdon, 2002). Thus, in a pathway without inhibitory receptor upregulation, there would be a ratiometric (i.e. direct) relationship between the extracellular input and intracellular output, provided that none of the pathway components are limiting. Studies of activin signaling in xenopus mesendoderm development are consistent with such a (comparatively straightforward) relationship, as ~3-fold increments of receptor occupancy lead to corresponding 3-fold increments of TF activation (Shimizu and Gurdon, 1999). Subsequent experiments indicated that, because the developmental window of competence to respond to signaling is shorter than the time for ligand dissociation, signal-regulated TF activation only increases with time (Bourillot et al., 2002).

By contrast, in a system that exhibits negative feedback regulation of extracellular input, a cell once exposed would not respond the same way to the same concentration of signal, such that increased ligand concentration would be required to maintain the same rate of TF activation over extended periods, provided that the concentration of ligand be (or become) limiting. This phenomenon, termed “temporal adaptation”, was observed for Shh signaling in the developing vertebrate nervous system by exposing explanted naive neural tissue to distinct but constant ligand concentrations, and monitoring Gli activity using a reporter driven by a multimerized Gli-binding site as a synthetic promoter (Dessaud et al., 2007). Interestingly, the Gli response over the first ~6h of exposure was found to be concentration-dependent only below 1nM of Shh, denoting a point at which receptors and/or transduction flow becomes saturating initially (Dessaud et al., 2010). Importantly, this period corresponded to the time required to reach an intermediate transcriptional response output (i.e. target gene expression) at saturating ligand concentrations (Dessaud et al., 2007), indicating that high responses can only be reached even at saturating levels by maintaining signaling for an extended period of time (Dessaud et al., 2010). The subsequent rate of return to baseline levels of TF activation would then depend on the rate of adaptation, e.g. *Ptch1* upregulation, such that a fast rate would result in a nearly simultaneous return to base-

line, whereas for a slow rate, cells exposed to high levels of signal would adapt more slowly than cells exposed to low thresholds. In the developing nervous system, explant data are consistent with the latter scenario. The initial inaccessibility of the highest response implies that the Shh-driven GRN exhibits hysteresis and feedforward regulation in order to reach that level of activation, a phenomenon that has been described following Shh exposure in both the developing limb bud and nervous system (Dessaud et al., 2007; Harfe et al., 2004). The underlying transcriptional mechanism has, however, been unclear.[32] This is addressed in **paper II**.

The Primary Cilium as an Assembly Line for Signal Transduction

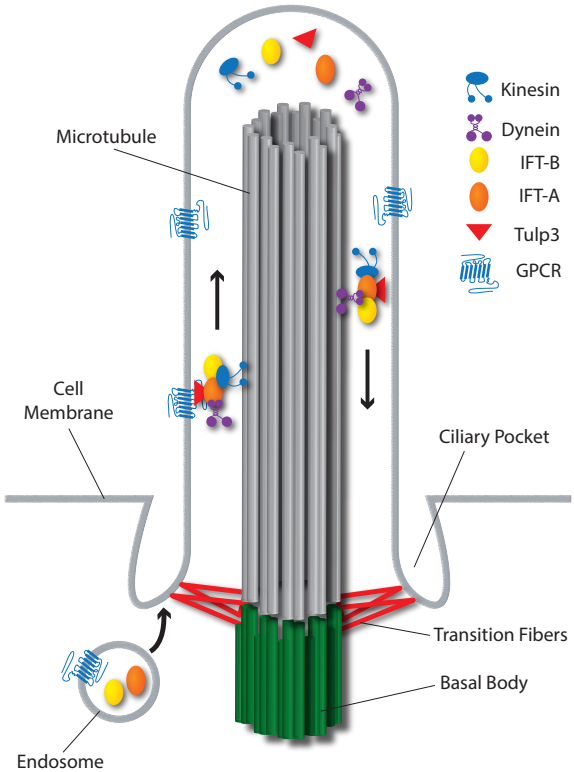

Figure 20. Structure of the primary cilium and intraflagellar transport. A complex of IFT proteins and their cargoes are transported by the endosomal pathway from the Golgi to the base of the cilium, where they are assembled into a complex that is transported along the ciliary axoneme by kinesin to the tip, whereupon they dissociate and reassemble to be transported down the axoneme by dynein. There are two IFT complexes, IFT-A and IFT-B, that are now thought to mediate different aspects of both anterograde and retrograde transport. Tulp3 is an IFT-A cargo that transports G protein-coupled receptors into the cilium.

The idea that signaling duration can be an important variable in regulating transcriptional responses in emphasizes that, as for any evolvable system subject to natural selection, speed can be an asset, and, as Henry Ford showed, productivity can be maximized by the use of an assembly line. Nature arrived somewhat earlier at the same conclusion, arraying regulatory components on cytoskeletal scaffolds, lipid rafts, or other structures that effectively limit diffusion distances, and compartmentalizing processes to increase the local concentrations of reactants (Freeman and Gurdon, 2002).

In vertebrates, nearly all cell types in the body carry a cilium, an organelle situated at the cell surface best known for governing sperm motility (reviewed in Nachury, 2014). Cilia are highly complex structures that are constructed as an extension of the basal body, one of the microtubule-organizing centers required for cell division. In non-dividing cells, the basal body is situated at the apical surface

of the cell, tethered by a protein mesh (called transition fibers) to the cell membrane, where it acts as a template for microtubule formation toward the cell membrane, forming an elongated bulge at the cell surface. These microtubules are arranged in a cylindrical conformation that, together with additional structural proteins, forms the axoneme. Generation and maintenance of the axoneme is thought to require new proteins to replace those that have been damaged, and the length of the cilium is dynamically regulated. Directed transport of these proteins along the axoneme is mediated by motor proteins and intermediaries, called intraflagellar transport (IFT) proteins, but transport in each direction along the axoneme requires distinct sets of motor proteins: kinesins mediate anterograde (tip-directed) and dyneins retrograde (base-directed) transport. IFT proteins are arranged in two complexes, IFT-B and IFT-A. Whereas kinesin and most IFT-B subunits are absolutely required for ciliogenesis, loss of dynein and IFT-A typically results in stunted, bulbous cilia in which IFT-B other proteins accumulate. This originally led to a model in which IFT-B and IFT-A mediate anterograde and retrograde transport, respectively, and consistent with this idea, cilia morphology and dysfunction in dynein and certain IFT-A mutants can be alleviated by genetic ablation of other single IFT-A subunits or core IFT-B subunits (Liem et al., 2012; Ocbina et al., 2011). Recent studies have painted a somewhat more complex picture, however, as the IFT-A complex binds and is required for IFT of Tulp3, a transporter of G protein-coupled receptors (GPCRs; Mukhopadhyay et al., 2010), whereas a subset of IFT-B mutants, in which cilia morphology is indistinguishable from wild type cilia, exhibit accumulation of some receptors (Keady et al., 2012). That being said, examination of the dynamics of individual receptor molecules indicate that receptors spend only ~25% of the time in the cilium in active transport, and then only for short distances, suggesting that IFT could act to facilitate their otherwise diffusion-driven distribution in the cilium (Nachury, 2014).

Beginning with the discovery that genetic ablation of IFT-B core proteins recapitulates the phenotype of *Shh* and *Smo* mutants (Huangfu et al., 2003), it has become increasingly clear that many signaling pathways are associated to some degree with the primary cilium. Subsequent studies have found that all dedicated components of Shh signal transduction are enriched in the cilium, and the ciliary localization of many of them is gated by Shh (figure 21; reviewed in Sasai and Briscoe, 2012). For example, ciliary localization of Ptch1 is normally required to suppress Shh signaling (Kim et al., 2015), and Ptch1 is endocytosed from the cilium in response to Shh stimulation, allowing Smo to enter (Rohatgi et al., 2007), which is required for pathway activation (Corbit et al., 2005). Shh signaling has been found to be dysregulated in all mutants that affect cilia morphology, and the effects of these mutations can be classed into four major groups: (I) core IFT-B mutants and anterograde motor mutants that are absolutely required for Shh signaling, although they are not as severe as *Shh* mutants (Huangfu and Anderson, 2005; Huangfu et al., 2003); (II) hypomorphs of some core IFT-B genes, mutants of *IFT25* and *IFT27*, and retrograde motor mutants with relatively mild or no ciliary morphological defects that merely fail to induce the highest Shh responses, with modest or no effects on mid- to low-threshold responses (Keady et al., 2012; Liu et al., 2005; May et al., 2005; Yang et al., 2015); (III) *IFT144* (of the IFT-A complex) mutants, as well as other factors including *Arl13b* and *Tmem107* in which the threshold of low and mid-range responses is lowered, but cells do not manifest the highest Shh responses (Caspary et al., 2007; Christopher et al., 2012; Liem et al., 2012); and (IV) other IFT-A and *Tulp3* mutants in which Shh signaling is constitutively active as a result, at least in part, of a failure of Tulp3 to transport the GPCR Gpr161 into the cilium (Mukhopadhyay et al., 2010; Mukhopadhyay et al., 2013; Norman et al., 2009; Qin et al., 2011). Gpr161 stimulates PKA to increase GliR by activating ciliary adenylate cyclases that locally increase cAMP levels which diffuse out to the basal body, where PKA is tethered (Mukhopadhyay et al., 2013). Whereas Gpr161 is endocytosed in response to Shh, a second cAMP-regulating GPCR, Adcyap1r1, is induced in response to Shh exposure, localizing to the cell membrane outside of the cilium,

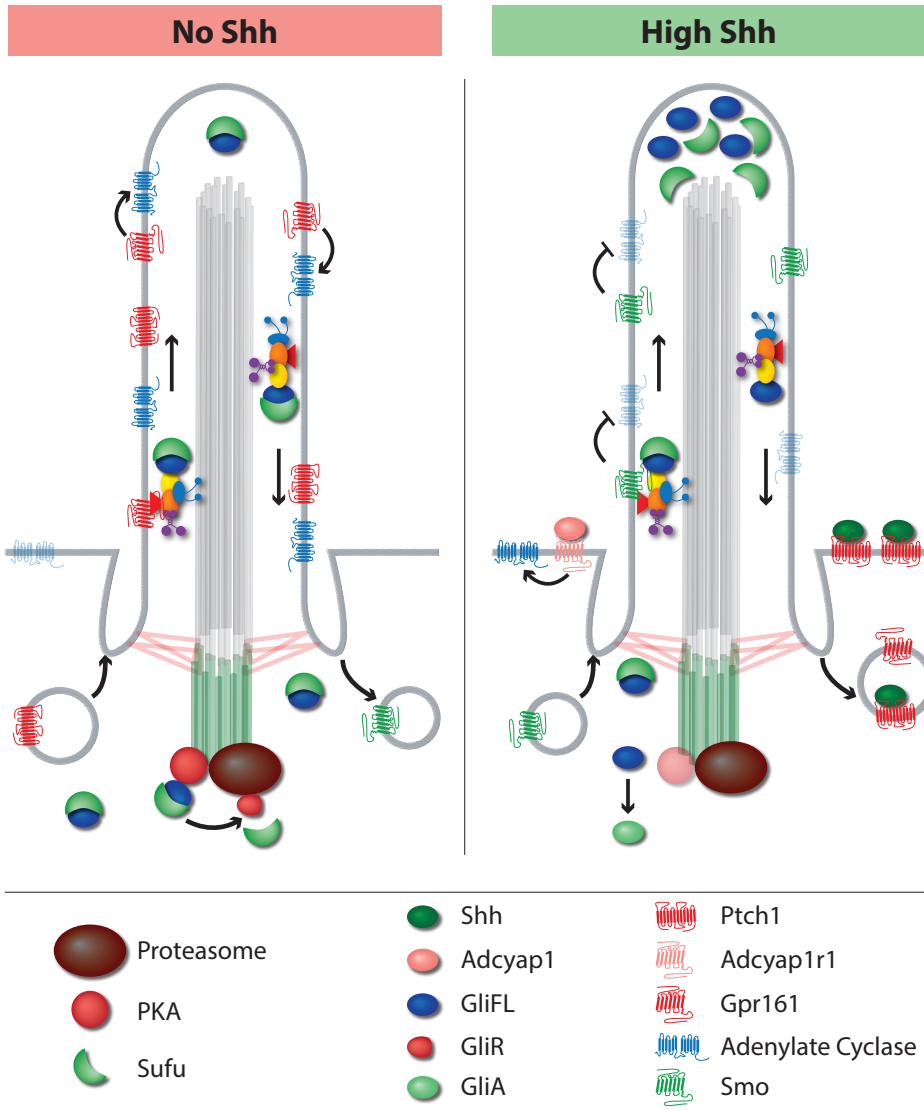

Figure 21. Shh signaling is transduced via the primary cilium.
In the absence of Shh, Ptch1 is localized in the cilium, where it catalytically inhibits ciliary localization of Smo. Gpr161 is also transported into the cilium by Tulp3 and the IFT machinery, where it activates adenylate cyclases that increase cAMP levels to stimulate PKA, which is localized at the basal body. PKA is thought to phosphorylate the fraction of Sufu-bound GliFL that has transited the cilium bound to IFT, and this leads to partial proteolysis of GliFL to GliR by the proteasome at the basal body. GliR then translocates to the nucleus, where it regulates transcription.
Upon binding of Shh, Ptch1 is internalized and this complex degraded by the lysosome. This allows ciliary accumulation of Smo and the concomitant exclusion of Gpr161 from the primary cilium, reducing the activity of adenylate cyclases, decreasing cAMP levels, and inhibiting PKA. In addition, GliFL becomes enriched in the cilium tip, where it is thought to dissociate from Sufu, allowing it to be modified as GliA, translocate to the nucleus and regulate transcription. Adcyap1r1 is induced by GliA and is thought to negatively regulate Shh signaling by stimulating PKA.

where it is thought to dampen Shh signaling by activating PKA[33] (Mukhopadhyay and Rohatgi, 2014).

Full-length, unactivated Gli proteins (GliFL) are found in the cytoplasm, where they are bound by Sufu. Sufu has three effects on Gli, (1) inhibiting nuclear translocation of GliFL, i.e. cilium-independent formation of GliA to maintain a cytoplasmic pool of GliFL for fast signal responsiveness; (2) competitively inhibiting nuclear degradation of GliFL; and (3) promoting GliR formation (Chen et al., 2009; Humke et al., 2010). Sufu-Gli are transported through the cilium in the absence of Shh, and production of normal levels of GliR is dependent on normal ciliary morphology (Huangfu and Anderson, 2005; May et al., 2005; Qin et al., 2011), but within minutes of Shh exposure, the flow of Sufu-Gli is strongly increased (Wen et al., 2010). In the presence of Shh, GliFL dissociates from Sufu at the ciliary tip, and this is thought to be a prerequisite for GliA formation. The precise location and mechanism of activation of GliFL has not been determined, but, like GliR formation, it does not occur properly in malformed cilia (Ocbina et al., 2011). The distinct Shh signaling phenotypes in cilia mutants correspond to distinct effects on GliFL[34] and GliR levels, as well as *Ptch1* and *Gli1* expression, which are used as readouts of Shh signaling that offer (limited) insight into the level of GliA, given that both are direct targets of the pathway (table; Dai et al., 1999; Lee et al., 1997).

	GliFL	GliR	*Ptch1*	*Gli1*
Smo	down	up	off	off
Ptch1/Sufu	down	none	up	up
Group I	up	down	off	off
Group II	up	down	down	down
Group III	n.c.	n.c.	mid	?
Group IV	down	none	up	up

Interestingly, numerous studies of vertebrate cilia indicate that ciliogenesis and function is regulated by a set of dedicated TFs of the Rfx family, as well as Foxj1 (Choksi et al., 2014). Foxj1 is specifically required to activate genes for motile cilia, whereas Rfx TFs regulate both motile and non-motile ciliary gene expression programs and exhibit a high degree of expression overlap and functional redundancy. In the developin CNS, for example, Rfx2-4 are co-expressed in neural progenitors and have highly similar DBDs and hence recognition site specificities, which has been proposed as an explanation of why the loss of individual factors does not result in a complete loss of cilia in these cells. Rfx4 mutant cilia, for example, resemble those of group II cilia mutants in morphology and Gli regulation (Ashique et al., 2009). In addition, however, many genes not involved in cilia function have been shown to be differentially expressed in Rfx4 mutants, raising the possibility that they may be directly involved in cell fate specification as well (Zhang et al., 2006), but this has yet to be established.

Morphogenesis and Regulation of Cell Identity Across Tissues

"*The father Somnus chose from among his sons, his thronging thousand sons, one who in skill excelled to imitate the human form; Morpheus his name, than whom none can present more cunningly the features, gait and speech of men, their wonted clothes and turn of phrase.*"

— Ovid, *Metamorphoses*

The discussion up to this point has largely centered on mechanisms by which an individual cell can interpret its environment, but this says relatively little about how groups of cells interpret their surroundings in relation to other cells in the embryo to generate the remarkably reproducible arrangement, or pattern, of cells in different specimens of a species or, indeed, of a phylogenetic clade. It comes as no surprise that the nature of the instructive mechanisms governing cell identity within a developing tissue are integrated with the mechanisms governing morphogenesis of that tissue.

Cells could be organized into tissues in several distinct, but often complementary ways. (1) Clumps of cells could be sculpted, e.g. by apoptosis of cells not needed in the mature tissue, which has the advantage that cells would not need to expend energy to get themselves or other cells to a specific location or restricting cell-cell contacts to certain surfaces of the cell, which requires establishing intrinsic asymmetry. Disadvantages associated with this strategy, however, include the lim-

ited potential for physiological exchange, the difficulty of determining cell identity in three dimensions, and that it is wasteful in other obvious ways. Nevertheless, this strategy is employed in many situations, notably digit formation (Gilbert, 2014). (2) Tissues could also be sculpted by compaction, i.e. selective regulation of cell adhesion and cytoskeletal reorganization that reshapes the morphology of constituent cells by mechanical constriction, as occurs in the transition from morula to blastula[35] (Takaoka and Hamada, 2012). (3) Cells can be reorganized and/or distributed by mass migration, the advantage being that their identity can determine their location, rather than the other way around, which may be useful when relatively small numbers of specific cell types need to be distributed through the embryo (related to the typological and topographical hierarchies discussed above). This could potentially involve delamination: the rapid loss of all previous cell-cell contacts, as in the case of the neural crest (Gilbert, 2014). By contrast, migratory cells are constantly in contact with neighboring cells in many regions of the gastrulating vertebrate embryo, reshaping themselves to mechanically push and pull other cells, collectively extending the length of the embryonic anteroposterior axis, but in this case a disadvantage is that tissue growth and morphogenesis are largely uncoupled, as the migratory cells do not divide[36] (Keller, 2012). (4) In another alternative (which could be called a Calzone strategy), cells grow as epithelial sheets, expanding outwards and folding into hollow tubes, spheres, or crypts that may be gradually filled as the tissue grows. An advantage of this is that cell identity can be initially coordinated over only two dimensions, a disadvantage being the parallel requirement for mechanical coordination of cells within the tissue and between tissues. This is the principle strategy used to build the CNS (figure 22; Gilbert, 2014).

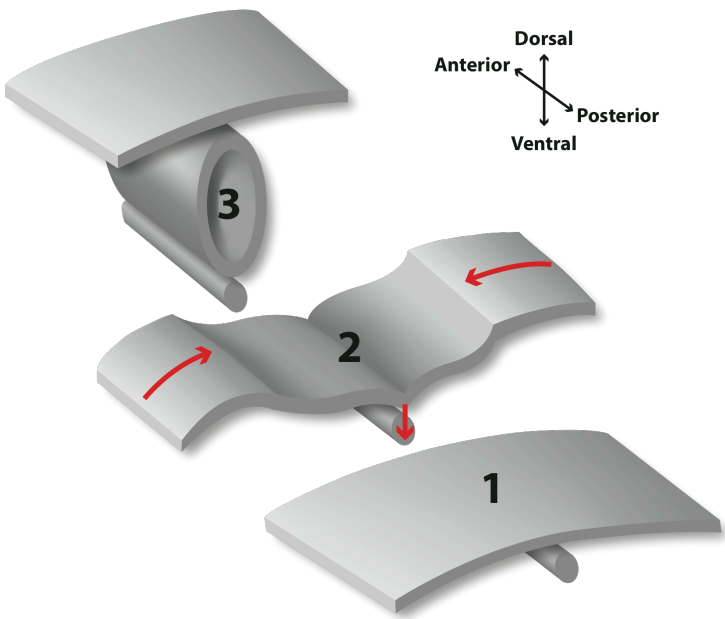

Figure 22. Morphogenesis of the developing CNS. The nervous system arises from the neural plate (1), which is pulled ventrally at the midline by the notochord and its edges pushed medially by the growth of the ectoderm to form the neural fold (2), and eventually forms a tube, delaminating from the overlying ectoderm (3). This process is not synchronized along the anteroposterior axis, so all stages can be viewed simultaneously just prior to midgestation in mouse.

It is conceivable that juxtacrine signaling or paracrine signaling alone could determine the identity and organization of all cells in a complex organism by relaying information between adjacent or nearby cells, but given that nearby cells can sense different concentrations of instructive molecules, it comes as no surprise that cells further away from the signaling source could respond to these diffusible molecules, provided that they be sufficiently diffusible. Such long-range signaling has a number of advantages, notably speed and the ability to redeploy such signals in different contexts to elicit distinct arrays of outputs. Before the molecular principles of signaling were understood, however, it was not obvious that this would be the case. The existence of gradients of some nature was inferred from a variety of regeneration experiments in which, for example, almost any piece of *Hydra* cut at both ends regenerates a head from the anterior end and a tail from the posterior end. However, if two pieces of different lengths are grafted together at the wrong ends, the shorter piece would reverse its polarity to regenerate a complete organism (Morgan, 1904). Nevertheless, it would be some time before a concrete mechanism was proposed for how graded, long-distance, information could be translated into pattern.

Patterning over Long Distance by Morphogens

"The key to the problem of pattern formation lies in the correct posing of the problem so that an answer can be obtained in terms of cellular behaviour."

— Lewis Wolpert, *Positional Information and the Spatial Pattern of Cellular Differentiation*

A confluence of mechanisms described above can be seen: that pools of stem cells in indeterminately developing organisms are initially equivalent, but competent only over a limited developmental window to respond to specific inductive signals, the activity of which in some cases appears implicitly to be graded in some way, resulting in polarization. The desire to understand how these processes are interconnected led to the idea that pattern formation can be instructed by the activities of morphogens. Morphogens may be defined as signaling molecules that diffuse across a tissue and can act over long distances from their sites of production in (or nearby) that tissue to regulate gene expression in an instructive manner as a result of graded exposure (Lander, 2007). As diffusible molecules, the range of morphogens depends on the steepness of the gradient across regions of high and low concentration, as well as the diffusibility of the molecules. It is worth noting that both models of morphogen activity initially arose as thought experiments.

Reaction-Diffusion

Perhaps not surprisingly, it was the (earlier) mathematically-defined model that invoked the amplification of stochastic differences leading to self-organization: a breaking of the symmetry of the embryo or tissue by a reaction-diffusion mechanism (figure 23; Turing, 1952). The basic idea is akin to (and can describe) the interactions between a predator and its prey, in which a diffusible activator (the prey) activates both itself and its own diffusible repressor (the predator). Due to stochastic fluctuations of the concentration of each morphogen, at some location, the activator will become sufficiently high that the repressor can no longer restore it to baseline levels. The prerequisite parameter is that the diffusibility of the repressor be greater than that of the activator, so that the repressor diffuses from the peak, allowing buildup of activator at the peak while repressing it laterally (reviewed in Green and Sharpe, 2015). Reaction-diffusion mechanisms offer an elegant means of generating repeated ("Turing") patterns that are non-identical across individuals, such as leopard spots and zebra stripes, depending on additional parameters. The periodicity of these patterns is determined by the wavelength generated by reaction-diffusion, which is dependent, at least in part, on the diffusivity of the morphogens. This also means that Turing patterns can only arise in tissues that grow uniformly. Despite its ingenuity as a symmetry-breaking mechanism, the Turing model is a completely unintui-

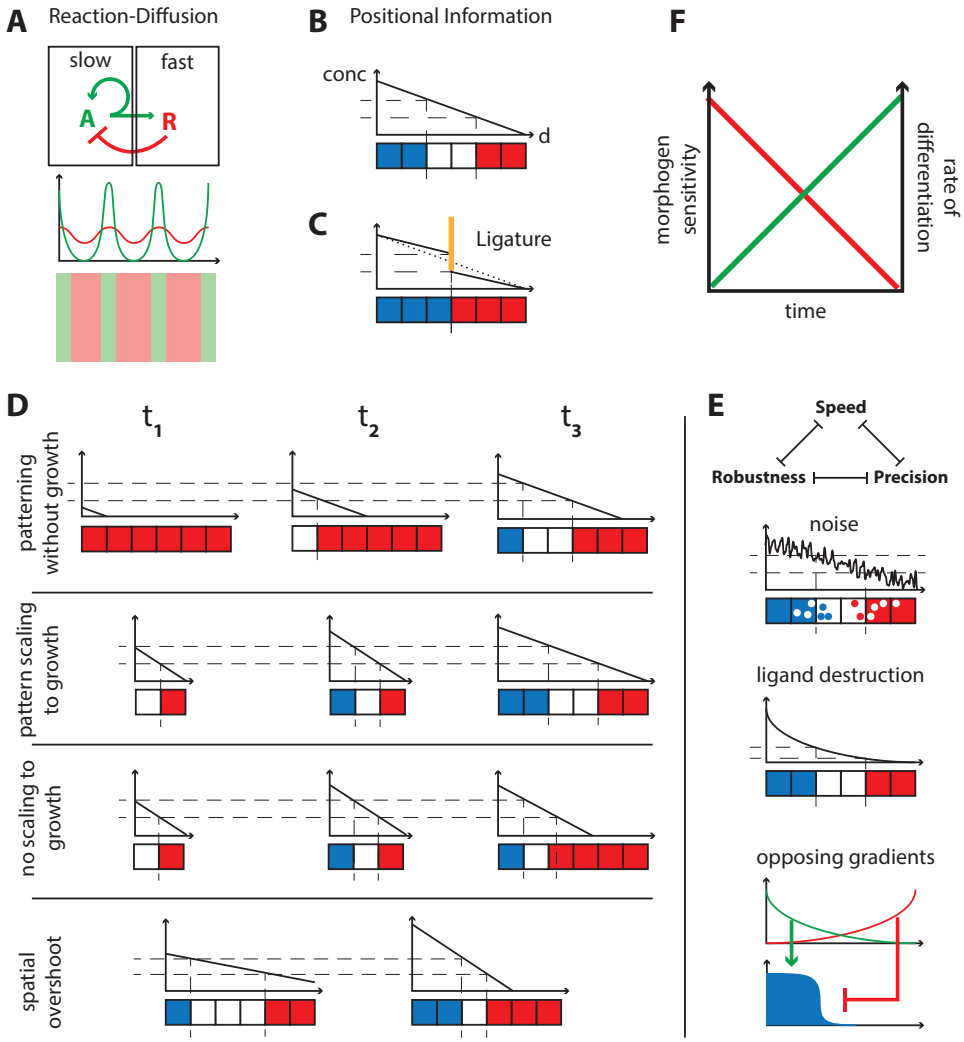

Figure 23. Pattern formation by morphogens.
(A) A Turing reaction-diffusion mechanism resulting in stripe formation (see text for details).
(B) The French Flag model in which positional information conveyed by a morphogen gradient induces target genes at distinct morphogen concentration thresholds.
(C) Embryo ligation mimics an increase in the steepness of the gradient, resulting in a loss of mid-threshold response and increase of low- and high-threshold responses.
(D) Temporal dynamics of cell responses to morphogen exposure at three time points. 1st row: As the concentration increases, more cells pass the response thresholds, so target genes are induced in temporal waves. 2nd row: in a uniformly growing tissue, domain sizes scale with overall tissue growth provided that the height and length of the gradient increase proportionately. 3rd row: stripes of different sizes can be created if pattern does not scale with growth. 4th row: upregulation of an inhibitory receptor over time gradually increases the steepness of the gradient by sequestering more ligand close to the source, effectively decreasing diffusivity.
(E) A mechanism that helps meet one performance objective compromises one or both others. Because diffusion is a stochastic process, the gradient is not smooth, so in the absence of compensatory mecha-
(*Continued on page 60*).

tive solution to a problem that, *a priori*, could be thought not to be a problem at all: there is still asymmetry between the inside and outside of the embryo, which could be disrupted deterministically by the buildup of a signal in the interior of the embryo more quickly than at its perimeter, where it would be released to the outside. Nevertheless, there is strong evidence of Turing mechanisms patterning mollusk shells as well as the vertebrate limb (Green and Sharpe, 2015). The major limitation of reaction-diffusion mechanisms, however, is the difficulty in imparting unique qualities to a subpopulation of patterned cells.

Positional Information

Embryological manipulations to study segmentation of insect embryos, which initially appeared an attractive candidate Turing pattern, eventually came down firmly on the side of a simple boundary-organized gradient in this particular system. Ligature experiments, in which the embryo is pinched at a defined spot for a defined period of time, resulted in the disappearance of segments that normally arise in the vicinity of the ligation site and concomitantly shifted the boundaries of the remaining segments toward the ligature site (figure 23; Sander, 1959 via French, 1988). Moreover, the earlier and/or longer the ligation was applied, the more segment identities were absent, implying that a gradient might be progressively established following fertilization. Subsequent loss-of-function studies, in which the anterior pole of the embryo was UV-irradiated, resulted in a failure to form the head and thorax, instead forming a symmetrical double-abdomen region, raising the possibility that this region corresponds to a morphogen source (Yajima, 1964). It was with this backdrop of axial polarity in regeneration and compartmentalization of body parts in insect segments that the "French Flag" model of pattern formation was born (Wolpert, 1969).

This intuitive idea was the result of a synthesis of observations that included and elaborated on those that gave rise to the morphogen concept, in particular, that (I) despite their indeterminate lineages, there are many instances of cells of the same functional class or type present in anatomically distant locations; (II) the normal consequence of equivalence is that only a fraction of the stem cell pool that could contribute to a tissue or cell type actually does so; (III) patterning is often invariant across individuals of a species and often even between species, irrespective of its size; (IV) development is epigenetic in the classical sense, (i.e. dynamic and progressive); (V) that morphogenetic fields occur in precise, invariant positions and times in the embryo; and (VI) that inductive events govern genetically specified processes, exemplified by the newt-tadpole ectoderm transplantations (Spemann and Schotte, 1932). These observations raised the possibility that (i) it is primarily the organization of cell typess within a tissue, rather than their subtype specialization, that determines the basic function of that tissue, but (ii) also implied that cell fate specification occurs with reference to the system as a whole, and (iii) indicated that positional information need not be directly coupled to differentiation.

According to the French Flag model, a concentration gradient is established across a tissue by diffusion of a morphogen from a source at one position within the tissue (or just outside it) to another end of the tissue that acts as a sink. This gradient enables cells to read out their coordinates along a one-dimensional space, eliciting different responses at distinct concentration thresholds. These responses consist of the expression of some quality, in this case, one of the three colors of the Tricolore. Positional information offered a simple explanation of many embryological findings, such as the

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nisms, sharp boundaries cannot be established. Ligand destruction generates an exponential curve that increases the slope of the gradient close to the source, resulting in sharp boundaries, but further from the source the slope is shallower, increasing sensitivity to noise. Opposing gradients can offset this problem, in part, but are only of limited efficacy, particularly in larger tissues.

insect ligature experiments: the ligature had the effect of increasing the slope of the gradient, such that cells close to the source are exposed to a higher concentration of morphogen, increasing the number of blue cells, whereas cells far from the source were exposed to a lower concentration, increasing the number of red cells.

Specification of the Anteroposterior Axis of the Drosophila Embryo

Positional information also provided a useful framework to make and test hypotheses. For instance, one of the implications of positional information is that the spatial order of specified regions is invariant: although cell types can be lost as a result of perturbation, their order cannot be scrambled (Wolpert, 1969). Thus, decreasing the rate of morphogen production would result in a greater number of cells producing low-threshold responses at the expense of high-threshold responses, i.e. red at the expense of white and white at the expense of blue (figure 23). This was consistent with the loss of head and thorax structures following irradiation of the anterior tip of the insect embryo, but a single gradient would not be sufficient to generate the mirror image of abdominal segments that resulted instead, implying the presence of a second gradient with its source at the posterior pole. This drew support from gain-of-function studies, in which the anterior cytoplasm of one insect embryo injected into the posterior pole of another could result in formation of a symmetrical head and thorax, while the inverse experiment resulted in a symmetrical abdomen (Nusslein-Volhard et al., 1987). Genetic studies in *Drosophila* identified *Bicoid* (*Bcd*) as a likely candidate for the anterior morphogen activity, as mutants of *Bcd* phenocopied the effect of anterior tip irradiation, which could be rescued by injection of anterior pole cytoplasm (Frohnhofer et al., 1986). The demonstration that Bcd is a *bona fide* morphogen required determination of its (i) expression, which was found restricted to the anterior tip of the embryo (Berleth et al., 1988); (ii) protein localization, which formed a gradient spanning most of the length of the embryo (Driever and Nusslein-Volhard, 1988); and (iii) the effect of increasing or decreasing protein levels (and consequently the slope of the gradient) on the spatial boundaries of expression of downstream genes, which was determined by gene dosage manipulation (Driever and Nusslein-Volhard, 1988).

Bicoid turned out to be rather unusual for a morphogen in that it is, itself, a transcriptional activator (Driever and Nusslein-Volhard, 1989), a phenomenon made possible by organization of the early *Drosophila* embryo as a syncytium. This made analysis of its transcriptional interpretation comparatively straightforward. After identifying a Bcd consensus binding site from sites identified in Bcd-regulated CRMs (Driever and Nusslein-Volhard, 1989), synthetic constructs, in which Bcd-binding sites of varying affinity were fused to a minimal promoter and reporter gene, were transposed into the Drosophila genome. As would be anticipated for a transactivator, high-affinity sites drove expression up to a greater distance from the source than lower-affinity sites (Driever et al., 1989). This finding gave rise to the affinity-threshold model of morphogen interpretation, in which binding site affinity is directly proportional to the range of target gene expression (figures 23, 25).

Patterning of the Dorsoventral Axis of the Developing Spinal Cord

Another prime candidate for boundary-organized pattern formation was the dorsal-ventral (DV) axis of the developing spinal cord, based on the ability of the notochord, an axial mesodermal tissue, to polarize the developing nervous system (Wolpert, 1969). This system had the advantage of being closely linked to terminal differentiation, providing a clearer readout of the regulation of specification and differentiation in response to morphogen signaling. Studies of this process demonstrated that the notochord induces a floor plate (FP) of ependyma-like cells at the ventral neural midline, as well as more laterally (distally) located cell types, including motor neurons (Yamada et al., 1991). Following the identification of *Shh* and the demonstration that its expression is restricted to the notochord

and FP, misexpression of Shh in the dorsal neural tube was found sufficient to induce an ectopic FP (Echelard et al., 1993). Direct evidence that Shh acts as a morphogen was contingent on the identification of additional markers of neural progenitor and differentiated neuronal subtypes, which showed that these subtypes are generated at stereotypic positions along the DV axis. These subtypes were shown to be sequentially induced in neural plate explants by ~3-fold increases[37] in the concentration of Shh (figure 24; Ericson et al., 1997; Roelink et al., 1995), satisfying the first criterion of a morphogen. Direct evidence that Shh patterns the nervous system over long distances was provided by misexpression of $Ptc^{\Delta loop2}$, resulting in cell-autonomous (i.e. in transfected cells) suppression of Shh-dependent gene expression, including *Ptch1*, as well as a concomitant ectopic activation of target genes at positions dorsal to their normal boundaries, due to a non-cell autonomous effect of the failure to sequester Shh in transfected cells (Briscoe et al., 2001).

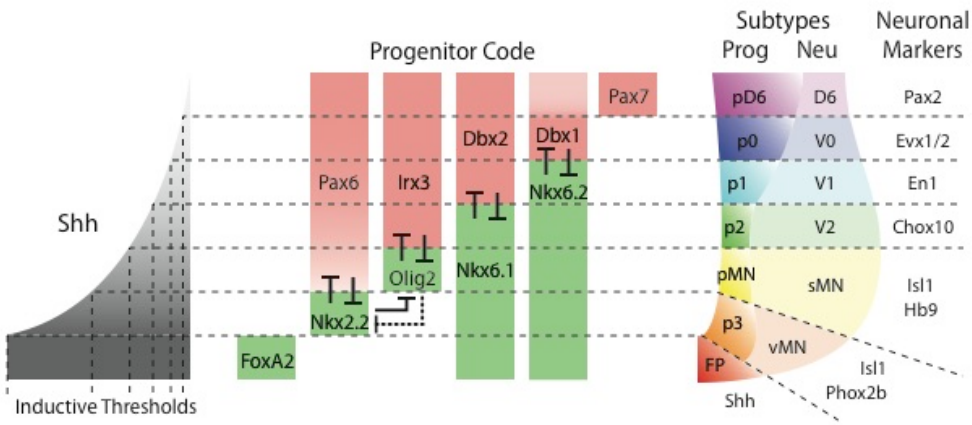

Figure 24. Ventral neural pattern formation by Shh
LEFT: A gradient of Shh emanating from the notochord (not shown) and subsequently FP results in the patterned expression of class I (red) and class II (green) TFs at distinct Shh concentration thresholds. MIDDLE: Class I and II TFs cross-repress each other in a pair-wise manner, resulting in a combinatorial TF code that specifies the distinct Shh-patterned progenitor domains of the neural tube (RIGHT).

Interpretation of Shh Morphogen Activity via Bifunctional Gli Proteins

The existence of three Gli paralogs in mice raises the question of whether these TFs regulate the same target genes, a problem addressed through competitive *in vitro* oligonucleotide-binding assays to define the consensus sequences of each Gli DBD, with each gene demonstrating nearly identical binding specificities (Hallikas et al., 2006). Another issue is whether Gli isoforms, i.e. GliA and GliR, differentially bind DNA, but no study has ever reported this to be the case in vertebrates or invertebrates. Given that Gli2 and Gli3 proteins mediating Shh signaling are bifunctional, an important issue is how Shh signaling affects the stoichiometry of GliA:GliR across the field of responding cells. Whereas a transcriptional activator like Bcd could regulate gene expression according to the affinity-threshold model, a repressor would be expected to repress genes that are active by default: an inverted affinity-threshold interpretation (figure 25). However, because Shh suppresses GliR formation, it has generally been assumed that inversely proportional, opposing intrinsic gradients of GliA and GliR are established over the region in which Shh signaling is active (e.g. Cohen et al., 2014), a not unreasonable notion for a tissue in which *Gli* genes would be uniformly expressed. Assuming that the basal level of expression, i.e. in the absence of either isoform, of all target genes be equal, the stoichiometric ratio of Gli isoforms

would be predicted to be interpreted along a continuum in which both GliA and GliR are instructive, the effect of GliR being to increase the threshold of signaling required to induce target gene activation, as well as to reduce noise (figure 25). A complication to this model is that *Gli* genes are *not* uniformly expressed in the neural tube: being a target of Shh signaling, *Gli1* expression is restricted to more ventral levels of the nervous system, whereas *Gli2* and *Gli3* become progressively downregulated in the ventral neural tube and upregulated more dorsally (Lee et al., 1997), making it difficult to infer the precise levels of Gli isoforms but apparently ruling out inverse proportionality and implying that target genes could be rather sensitive to the level of GliR. Genetic ablation of all Gli activity in *Gli2;Gli3*$^{-/-}$ double mutants[38], however, revealed that only the most ventral cell domains, the Foxa2^{+} FP and Nkx2.2^{+} p3 domain, were absent, whereas the dorsal limit of expression of low- and mid-threshold response genes was broadly similar to wild type animals (Bai et al., 2004; Lei et al., 2004), indicating that each target gene interprets the same Gli gradients differently, a phenomenon called pre-patterning. Nevertheless, subsequent gain-of-function studies, in which an obligate activator form of Gli3 was misexpressed in the developing chick CNS, indicated that activation of Shh target genes is proportional to the level of GliA, consistent with an affinity-threshold interpretation, and resulting in a commonly represented model in which the GliA gradient is directly interpreted and GliR merely suppresses expression in the dorsal neural tube (figure 25; Stamataki et al., 2005), but raising the question of what useful purpose GliR could serve, given that target gene repression would not be necessary. Experiments to address these issues are presented in **paper I**.

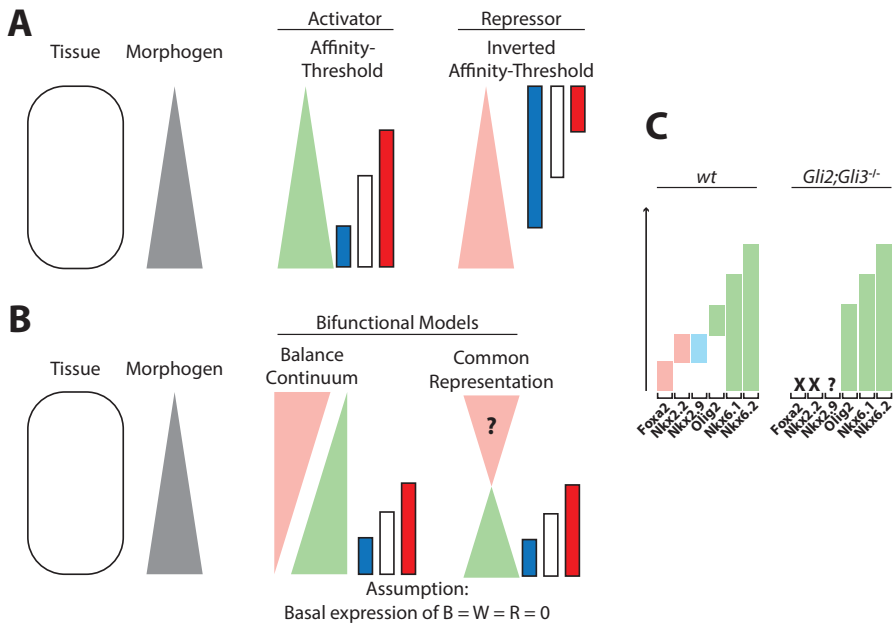

Figure 25. Models of the transcriptional interpretation of morphogen signaling.
(A) Interpretation mediated by an obligate activator or repressor TF. The concentration of active TF mirrors the morphogen gradient and the range of target gene activation or repression is directly proportional to the TF-binding site affinity (red = hi-affinity; white = med-affinity; blue = low-affinity).
(B) Interpretation mediated by a bifunctional TF. Balance model: all target genes directly interpret the balance between activator and repressor isoforms, and range of expression is determined by TF-binding site affinity. It is commonly assumed that basal expression of all targets = 0. An alternative model is also depicted in which morphogen interpretation follows the affinity-threshold model, with the repressor providing additional repressive input at dorsal levels, though why this would be useful is unclear.

Dynamic Establishment of Subtype Identity and Regulation of Growth

Being diffusion-driven, the establishment of a concentration gradient is a dynamic process — Rome wasn't built in a day — so if the tissue were responsive throughout this period, target gene initiation, and hence cell type specification, would occur in successive temporal waves originating near the source and traveling outward with the expansion of the gradient, in order of low- to high-threshold responses (figure 23). By contrast, the initiation of differentiation would be predicted to be more-or-less simultaneous, as the steady state expression pattern at each position would be reached at roughly the same time. If only it were so simple. In the *Drosophila* syncytium, temporal expression mapping of the Bcd-regulated gap genes indicates that the distal boundaries of target genes are already roughly at their steady state positions upon induction[39] (Wotton et al., 2015), whereas although target genes of each morphogen in the developing spinal cord are indeed activated in sequential waves (Diez del Corral et al., 2003; Jeong and McMahon, 2005; Tozer et al., 2013), among Shh-regulated cell types, it is the ventralmost (the FP) that differentiates first in this tissue (Placzek et al., 1990).

Various factors could contribute to this behavior in the developing CNS. The most obvious is that factors other than Shh govern the timing of differentiation: for example, expression onset of the neurogenic TFs that drive differentiation is regulated by Fgf and retinoid cascades (Diez del Corral et al., 2003). Secondly, because of temporal adaptation and the increased sequestration of Shh by Ptch1, the steepness of the Shh gradient is predicted to increase over time (Chen and Struhl, 1996), such that more distal cells initially receive more signal than they do at later time points resulting in respecification of some cells. Lineage tracing of neural cells that have previously expressed either a more proximally expressed target gene of Shh or RA have provided evidence of such a spatial overshoot (figure 23; Dessaud et al., 2010). Given these dynamics, it is likely that the gradient never reaches a steady state over the window of competence to respond (Nahmad and Lander, 2011). A possible consequence could be that cells not in contact with or very near to the morphogen source would require more time to accurately gauge their positions and commit to a program of differentiation in the fluctuating signaling environment, an issue examined in **paper III**.

The complexity of these dynamics is compounded by the concurrent growth of the tissue, because increases in tissue size increasingly dilute the morphogen. This is dealt with in different ways in different systems. In the conceptually simplest system, once the pattern is established, it scales up with tissue growth. Assuming that cells would continue to be sensitive to the level of morphogen exposure during at least a part of this time, the concentration of the morphogen across the tissue must increase such that both the height and length of the gradient increase (Fried and Iber, 2014). However, it would also be possible for domain size to be uncoupled from the rate of growth, and instead be timed to produce the correct proportion of postmitotic progeny. Recent studies demonstrate that although pattern in the developing nervous system scales between organisms of different sizes and species, domain sizes do not scale to tissue size over time within an organism, but rather exhibit biphasic growth, in which progenitors are first dynamically specified in response to morphogen exposure and later differentiate at subtype-specific rates (Kicheva et al., 2014). Interestingly, both the propensity of neural subtype progenitors to be re-specified due to perturbation of morphogen signaling and the rate differentiation change as a function of time, and appear to be inversely correlated (Kicheva et al., 2014), consistent with the possibility that these processes may be mechanistically coupled, although how this switch between phases could be regulated is unclear.

It seems like a lot of trouble to go through to ensure that a small population of cells at the ventral midline differentiates first, but the FP is quite different from other ventral neural subtypes, with its constricted, wedge-shaped cell morphology, slow cell cycle, and non-neurogenic competence (Placzek and Briscoe, 2005). Importantly, tissue grafting experiments showed that the FP can substitute for the notochord in the polarization of the neuroepithelium, including the induction of an ectopic FP (Yamada et

al., 1991), a phenomenon termed homogenetic induction (Spemann, 1938). The efficiency of homogenetic induction declined with the age of the neural explants, implying a loss of competence over time (Placzek et al., 1993). Subsequent experiments indicated that this activity is due to the secretion of Shh from the differentiated FP (Echelard et al., 1993), and recent studies in which Shh was conditionally ablated in the FP demonstrated that it is required to maintain progenitor domains, with more dorsal domains exhibiting increased sensitivity as compared to more ventral domains, consistent with the dynamics of temporal adaptation (Dessaud et al., 2010). The secretion of Shh from these cells raises the issue, however, of how the FP can be constrained to the ventral midline, given that neural tissue is therefore continually exposed to high levels of Shh, first from the notochord and then from the FP. A recent study of FP differentiation has suggested that the duration of Shh exposure is a critical parameter governing FP commitment. Interestingly, however, this required the inhibition of Gli-dependent Shh signaling, as misexpression of either full-length Gli2 or an obligate Gli2 activator variant was sufficient to suppress FP differentiation (Ribes et al., 2010). If temporal adaptation were sufficient to prevent progressive ventralization beyond a certain point on the DV axis, it would require faster adaptation to high levels of signaling dorsally than ventrally, in which case the competence to respond to Shh would need to be restricted in a Shh-independent manner. In fact, it is unclear whether graded differences in Shh exposure are sufficient to account for the selection between a FP fate and that of the p3 progenitors dorsally abutting it, as induction of the two fates has been difficult to segregate genetically in Gli, Shh pathway, or cilia mutants (Bai et al., 2004; Litingtung and Chiang, 2000; Liu et al., 2005; Matise et al., 1998; May et al., 2005; Wijgerde et al., 2002). Moreover, apart from Gli, no transcriptional suppressor of FP fate has been previously described. These issues are explored in **papers II** and **III**.

Precision in Morphogen-Dependent Boundary Formation

Although the morphogen gradient of the original French Flag model was depicted with a linear slope, it is recognized that there is some degree of decay in morphogen gradients, e.g. due to receptor internalization and tissue growth. In other words, the gradient is more-or-less exponential, becoming shallower as a function of distance from the signal source (figure 23). In a deterministic system, this would not be a problem; however, due to stochastic fluctuations in the concentration of morphogen as the molecules perform their random walks across the tissue, cells at more distal regions will have greater difficulty reading out their coordinates. One way to address this problem is to deploy a second gradient at the opposite end of the tissue, such as *nanos* at the posterior pole of the *Drosophila* embryo, which represses Bcd target genes to create sharp boundaries that are critical in the compartmentalization of the embryo (Irish et al., 1989). The developing spinal cord goes further, deploying not only the GliR gradient, but also Bmps and Wnts from the roof plate at the dorsal midline to pattern the dorsal spinal cord, and paraxial mesoderm-derived RA that drives target gene expression at intermediate levels of the DV neuraxis (Le Dreau and Marti, 2013; Novitch et al., 2003; Pierani et al., 1999). Other factors, however, exacerbate it, such as *Ptch1* upregulation, which reduces Shh diffusivity (Lander, 2007), as do sulfatases, which are expressed in the ventral neural tube and regulate the extracellular matrix through which Shh diffuses (Danesin et al., 2006). Each of these mechanisms has an optimal performance range that must be reconciled with the others in the system (Lander, 2007), but with so many extrinsic mechanisms to influence the ability of cells to respond to a gradient, an important question is how effective they are, in aggregate, at limiting the effects of noise. This may seem an odd way of looking at it, given that the system clearly achieves its performance objectives or there would be no one to write this thesis. But whether *graded* information achieves the required precision for noise cancellation, or whether other mechanisms intrinsic to the responding cell and/or GRN architecture are required to produce sharp boundaries, is the subject of **papers I**-I**II**.

Studies of *Drosophila* offer insights into morphogen interpretation at gene regulatory levels that provide a basis for further examination. Initial analyses of Bcd-regulated CRMs identified clusters of Bcd-binding sites in a *Hb* CRM, and increasing the number of Bcd-binding sites in a synthetic promoter increased the sharpness of the border of expression as well as the range of induction, leading to the suggestion that binding site number is a key determinant of the level of target gene expression (Driever et al., 1989). The aforementioned studies of *Eve* regulation provided evidence that other activators regulate distal target genes in synergy with Bcd, whereas repressors can delimit their posterior borders (Small et al., 1991). Comparative mutational analyses of a number of Bcd-regulated CRMs found that neither binding site affinity nor number of binding sites per cluster correlated strongly with positional boundaries, arguing against a simple affinity-threshold interpretation of morphogen activity; indeed, Bcd appeared to be the primary determinant only of the subset of CRMs with activity restricted to anterior regions, but in these there was a tendency for affinity to be lower, reminiscent of the affinity-threshold model (Ochoa-Espinosa et al., 2005). Whether this gene regulatory logic applies to other morphogens and other species has been unclear; for Shh-dependent patterning of the spinal cord, this stems partly from the fact that, apart from *Foxa2* (Sasaki et al., 1997) and *Nkx2.*2 (Lei et al., 2006), direct, neural-specific targets of the pathway have not previously been determined. These issues are addressed here primarily in **papers I** and **III**.

Robustness of the GRN Regulated by Repressive Interactions Between Morphogen Target Genes

Repressive mechanisms are a tacit implication of the stripes of the French Flag model: in a simple network in which the morphogen is the sole input for each target gene, one would predict that the red field would completely encompass both the blue and white fields, and the white field the blue, as there are no repressive constraints. In order to see a red stripe, there must be repression mediated by some TF(s) in the blue and white regions, probably induced by the morphogen. In the developing nervous system, a number of genes repressed or induced in response to distinct thresholds of Shh exposure, called class I and II genes, respectively, have been found to encode TFs. Each member of these classes is expressed in a domain with a boundary juxtaposed to that of a member of the other class, and, through loss- and gain-of-function analyses these pairs have been shown to exhibit cross-repressive interactions, forming a network of double-negative motifs that establish and maintain their common boundaries (figure 24; Briscoe et al., 2000; Briscoe et al., 1999; Ericson et al., 1997; Novitch et al., 2001). It is worth emphasizing that, in the conventional view, expression of class I and II genes is therefore viewed as mutually exclusive. In addition, the ventral boundaries of expression of the class II genes *Nkx2.2*, *Nkx2.9*, and *Olig2* do not extend to the ventral midline, and instead form stripes, and in the case of *Olig2*, gain-of-function studies have shown that this ventral repression is mediated, at least in part, by Nkx2.2 (Novitch et al., 2001). Genetic ablation of *Olig2* has shown that it, in turn, acts as a weak repressor of *Nkx2.2*, in conjunction with Pax6, as well as of Pax6 (Balaskas et al., 2012; Zhou and Anderson, 2002). By contrast, expression of the class II genes *Nkx6.1* and *Nkx6.2* extends from the intermediate neural tube to the ventral midline. The partially overlapping expression of class I and II TFs results in a combinatorial code of transcriptional activity that collectively establishes progenitor subtype identity within each of the five resulting progenitor domains (figure 24; Briscoe et al., 2000). Underscoring the importance of cross-repression in morphogen interpretation, genetic ablation of cross-repressive partners results in derepression of its counterpart throughout the adjacent domain (Briscoe et al., 2000; Briscoe et al., 1999; Ericson et al., 1997; Zhou and Anderson, 2002). Moreover, gain-of-function experiments with chimeric EnR- or VP16-containing variants of the patterning TFs showed that, with the exceptions of Pax6 and Irx3, patterning and cell fate specification by these proteins is achieved by Tle-dependent transcriptional repression mediated by Tle-interacting domains in the repressor TFs (Muhr et al., 2001). Neural progenitor

subtype specification is therefore sometimes described as a strategy of derepression of alternative fates in which patterning TFs act permissively, but it is important to note that a number of activators, e.g. Pax6 and Phox2b, are also expressed along the DV axis of the caudal nervous system and play important roles activating differentiation drivers such as *Ngn2* and *Ascl1*, respectively (Bel-Vialar et al., 2007; Dubreuil et al., 2002).

The mechanisms by which Pax6 and Irx3 indirectly repress *Nkx2.2* and *Olig2* are not well understood[40]. Irx3 has been shown to regulate a microRNA that triggers degradation of *Olig2* transcripts, but genetic ablation of this microRNA results in only partial derepression of *Olig2* within the p2 domain (Chen et al., 2011), suggesting that other mechanisms could also be involved, given that loss-of-function mutants of other patterning TFs result in expansion throughout the entire adjacent domain. With respect to Pax6, the data are conflicting: *Tcf7l2*, the transcriptional mediator of Wnt signaling, has been reported to be partially downregulated in response to genetic ablation of *Pax6*, and analysis of a CRM for *Nkx2.2* identified Tcf-binding sites that were required in a transgenic mouse line to prevent ectopic reporter expression throughout the intermediate neural tube, suggesting that Tcf7l2 mediates repression of *Nkx2.2* downstream of Pax6 (Lei et al., 2006); however, gain-of-function studies with wild type, dominant negative, and obligate repressor/activator variants of Tcfs provide compelling evidence that Tcfs, like Pax6, act as activators in constraining *Nkx2.2* expression in response to Wnt/ß-catenin signaling (Alvarez-Medina et al., 2008). This activity appears to be mediated, at least in part, by upregulation of *Gli3* (Alvarez-Medina et al., 2008). The role of Pax6 is studied further in **paper II**.

Cross-repression has generally been described as a mechanism for generating and maintaining sharp boundaries, because it results in all-or-none, bistable states (e.g. Balaskas et al., 2012; Briscoe et al., 2000). However, by buffering the GRN to ongoing fluctuations in signaling, cross-repression in the absence of a mechanism to achieve spatial averaging would result in boundaries that reflect the noise of the gradient. Cell sorting has been proposed to contribute to sharp boundary formation in the zebrafish nervous system (Xiong et al., 2013), but this cannot explain the re-specification of progenitors that follows the spatial overshoot observed by lineage tracing for both class I and II target genes (Dessaud et al., 2010). This problem is studied in **paper III**.

Transcriptional Integration of Positional Information Across Multiple Axes

Given the three dimensions of the organism that must be coordinately patterned, it is conceivable that the coordinate system of positional information could be extended to all three dimensions by directly integrating the information provided by three or more morphogens (i.e. at least one per axis) into the gene regulatory elements of individual target genes, resulting in the activation of each in a small volume of the three dimensional space, like the individual units of an apartment block. However, morphogens only provide effective positional information over tens of cells, which relates to the range of possible and effective gradient slopes (Wolpert, 1969), making it difficult to specify the components of body parts in an indeterminate lineage without prior compartmentalization and hence a need for additional morphogens. Perhaps more importantly, with gene duplication the primary source of evolutionary adaptability, it would limit the scope for re-wiring of the GRN that regulates the body plan to inputs from the morphogen and necessary cross-repressive interactions, making it difficult to modulate the size of body parts or cell populations, as well as to alter morphology and pattern.

Instead, the DV neuraxis expresses the same class I and II genes in the same pattern throughout much of the CNS, whereas the AP neuraxis is patterned by a separate set of TFs, first discovered as a result of their regulation of the segment identity of the *Drosophila* body plan (Gilbert et al., 1996). The most famous of these, *Bithorax*, was so named because of loss-of-function mutations resulting in a posterior-to-mid-thorax conversion of segment identity, including an extra pair of wings (Lewis, 1978).

The genetics of similar such phenotypes led to the current understanding that insect segment identity is regulated by eight genes that arose by duplication of an ancestral TF-encoding gene that governed a mid-Thorax-like identity and from which all other segment identities have since diverged (reviewed in Gehring et al., 2009). Strikingly, the *Hox* gene cluster, as it came to be known, is functionally and architecturally conserved across eumetazoa and heralded the Cambrian explosion (Gehring et al., 2009), although the entire cluster has been duplicated twice in amniote vertebrates since the last common ancestor with arthropods, is regulated rather differently across bilaterians, and oversees segmentation in mammals that is rather differently from *Drosophila* (Gilbert et al., 1996). Based on genetic linkage mapping, it was proposed that *Hox* genes are collinearly expressed and arrayed on the same chromosome, such that their order on the chromosome dictates the temporal and spatial order of onset along the AP axis, whereby the furthest downstream gene is the first to be activated and is (at least initially) the most broadly expressed (Lewis, 1978). Initially based on genetic loss-of-function and epistasis studies in *Drosophila*, a model of "posterior prevalence" was proposed, in which the activity of the most recently initiated Hox gene is dominant and consequently dictates segment identity. However, an alternative combinatorial model has been proposed in which Hox TFs would have unique functions in segment identity (reviewed in Alexander et al., 2009). This is consistent with the ability of Pbx and Meis TFs to modify the DNA-binding specificity of Hox genes via their interactions with linear motifs (Slattery et al., 2011).

In the vertebrate nervous system, the hindbrain is segmented into rhombomeres, the identities of which are regulated by the anterior group of the four hox clusters (Hox1-4 Alexander et al., 2009). Hox genes are induced in the neuromesoderm by opposing gradients of Fgf and RA (Alexander et al., 2009), which, remarkably, means that the same morphogens pattern both the AP and DV axes of the nervous system. A salient issue is how transcriptional regulators specified along these axes coordinate cell identity at transcriptional levels. Genetic evidence indicates that there is significant inter-regulation: for example, *Nkx6* genes are required to maintain expression of *Hoxb1* in the p3 domain of r4, and *Hoxb1* in turn maintains expression of the Shh target gene, *Phox2b*, which is required for the prolongation of vMN generation (Pattyn et al., 2003a; Samad et al., 2004). However, the *cis*-regulatory evidence for direct cross-regulation has hitherto been limited. Given that the generation of certain DV-specified subtypes, including sMNs, is discontinuous along the AP axis (Pattyn et al., 2003b), it is possible that at least some cell types are specified via lineage modules deployed in genealogically unrelated locations such as those found in *C. elegans* (Azevedo et al., 2005), and in which DV input would play a modulatory role in identity. If this were the case, one would anticipate that the Hox code regulates the competence of each rhombomere to generate classes of neurons. In a simpler alternative model, the discontinuous generation could be due to the differential modulation of DV-specified programs by AP inputs, a mode of action previously associated with Hox TFs (Davidson and Erwin, 2006). This issue is studied in **paper III**.

Another important question is whether multiple morphogens directly and coordinately regulate any of the same target genes at the same time and place, which intuitively seems probable. The finding, for example, that *Olig2* can be induced by RA in neural explants in the absence of Shh is consistent with such a possibility (Novitch et al., 2003). Similarly, it is unknown how cross-repression between class I and II TFs is orchestrated at *cis*-regulatory levels, though clearly in the case of Pax6 and Irx3, at least, this activity must be indirect. Nevertheless, there is strong *cis*-regulatory evidence both in *Drosophila*, downstream of morphogens such as Bicoid as well as in the Haematopoietic system that cross-repression is direct in these systems (e.g. Laslo et al., 2006; Small et al., 1991), raising the possibility that at least a subset of neural patterning TFs could operate in this manner as well. The direct integration of morphogen inputs, as well as of morphogen-regulated inputs into the GRN governing neural subtype identity along the DV and AP axes is studied in **papers III** and **IV**.

The Tissue-Specific Interpretation of Positional Information

Expression of the morphogen-regulated class I and II genes is restricted to CNS, consistent with the "habits of highly effective signaling pathways" (Barolo and Posakony, 2002). The observation that all major signal-regulated TFs seem to require the synergistic activity of accessory TFs raises the question of whether generalizable rules also apply to the selection of such factors to be integrated into the morphogen-regulated GRN. In particular, one would anticipate that they would be network hubs, limiting the potential for mutations that could disrupt the network. According to this logic, these hubs would be predicted to be broadly expressed in the responding tissue, limiting the number of inputs into each CRM required to permit a complete and appropriate response to morphogen exposure, and, moreover, would regulate many of the morphogen target genes. Soxb1- and Pou3-class TFs are broadly and selectively expressed in neural progenitors and required for neural identity (Bylund et al., 2003; Josephson et al., 1998), and previous in silico analyses indicate that they are enriched in the vicinity of neural-specific Shh target genes (Bailey et al., 2006), raising the possibility that either or both classes could act to regulate the neural-specific interpretation of Shh and other morphogens that pattern the vertebrate CNS. This is explored in **papers I** and **IV**.

AIMS

Using the regulation of neural subtype specification by Sonic hedgehog singaling in the developing vertebrate nervous system as a model of pattern formation by morphogens, this thesis aims

- to explore the implications of morphogen interpretation mediated by a bifunctional transcription factor for target gene regulation;
- to examine the potential for mechanistic differences between morphogen interpretation at short and long range and the extent to which such differences could affect the response to dysregulated morphogen signaling;
- to understand the gene regulatory logic underlying
    - the tissue-specific response to broadly active morphogen signaling pathways;
    - the integration of positional information encoded by opposing gradients and gradients acting on different embryonic axes;
- to examine the gene regulatory mechanisms by which ongoing morphogen interpretation could influence subsequent morphogen interpretation to establish pattern that is both robust and precise over a suitable timeframe;
- to explore the mechanisms by which patterning and growth become uncoupled in the developing nervous system;
- to understand the link between spatial pattern formation and temporal regulation of progenitor competence and cell fate commitment.