Pioneer transcription factors shape the epigenetic landscape

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Pioneer transcription factors have the unique and important role of unmasking chromatin domains during development to allow the implementation of new cellular programs. Compared with those of other transcription factors, this activity implies that pioneer factors can recognize their target DNA sequences in so-called compacted or "closed" heterochromatin and can trigger remodeling of the adjoining chromatin landscape to provide accessibility to nonpioneer transcription factors. Recent studies identified several steps of pioneer action, namely rapid but weak initial binding to heterochromatin and stabilization of binding followed by chromatin opening and loss of cytosine-phosphate-guanine (CpG) methylation that provides epigenetic memory. Whereas CpG demethylation depends on replication, chromatin opening does not. In this Minireview, we highlight the unique properties of this transcription factor class and the challenges of understanding their mechanism of action.

In the late 1970s when chromatin structure was beginning to be probed with tools such as DNase (hyper)-sensitivity, the concept of pioneer factors emerged. These were factors that would have the capacity to bind specific DNA sequences within compacted heterochromatin and initiate the opening of this chromatin. This opening would be required for implementation of major developmental fate decisions. At the same time, *Drosophila* geneticists developed the notion of selector genes for early developmental regulators that in some way specify the outcome of future cell fates through their action on broad embryonic domains (1). In this context, the notion of pioneer factors offered a possible mechanism to achieve the purpose of selector genes, but these ideas remained more in the domain of evening conversations than experimental reality. For clarity, it should be mentioned that in more recent years, the term "selector" has been used by some to identify factors that have the opposite effect in the differentiation scheme compared with the original definition, namely factors that trigger the ultimate step in cell-fate decisions (2).

The idea of pioneer action was revived in the late 1990s when the transcription factor (TF)[2] FoxA was shown to have the unique ability to bind its target sequence within nucleosomal DNA (3). This unique ability contrasted with many other TFs that will only bind efficiently naked or more readily accessible DNA as observed within active regulatory sequences. However, pioneer factors do not have completely unrestricted access to heterochromatin sites but do exhibit cell-specific actions (4). In parallel, the old binary view of chromatin as either hetero- or euchromatin changed dramatically as the enormous diversity of histone modifications became known, eventually leading to the concept of a histone code (5) that defines a continuum of chromatin flavors associated with regulatory and structural functions. The complexity of this code and the limited tools available to characterize chromatin limit our present ability to define the permissive or restrictive chromatin states that are targeted by pioneer factors. Despite this limitation, the basic features that define pioneer factors (Fig. 1) are as follows: 1) the ability to bind specific DNA sequences within "closed" or unmarked chromatin where genomic DNA is not readily accessible; 2) the ability to initiate chromatin remodeling leading to DNA accessibility; 3) consequently to allow binding of other transcription factors; and 4) finally to establish stable changes in chromatin structure associated with DNA accessibility and epigenetic stability. Collectively, these features imply that the "act of pioneering" may be a one-shot affair, *i.e.* once enacted, its effect on chromatin remains stable. Mechanisms for maintenance of chromatin state at pioneered sites may also exist. This Minireview will discuss the unique aspects of pioneer action and attempt to separate these from the transcriptional actions of the same factors because pioneers do act as transcriptional regulators like other TFs and often at the pioneered as well as other target sites. The list of TFs that share at least some features of pioneers is provided in Table 1.

Because the measure of chromatin features such as DNA accessibility and chromatin marks are not just absent *versus* present but are also present on a continuous scale, the expectation of pioneer function must be more clearly defined. Indeed, DNA accessibility (whether measured by DNase sensitivity (6), formaldehyde-assisted isolation of regulatory elements (FAIRE (7)), or the ATAC procedure (8)) or histone marks, such as histone H3K4me1 that marks active enhancer sequences (9), exhibit greater values as the activity of enhancers or the number of bound TFs increases (10). Increments in these marks may reflect quantitative changes in enhancer activity rather that the

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[2] The abbreviations used are: TF, transcription factor; TSS, transcription start site; iPS, induced pluripotent stem; FAIRE, formaldehyde-assisted isolation of regulatory elements; ER, estrogen receptor; AR, androgen receptor; ES, embryonic stem cell; CpG, cytosine-phosphate-guanine; C/EBPα, CCAAT/enhancer-binding protein α.

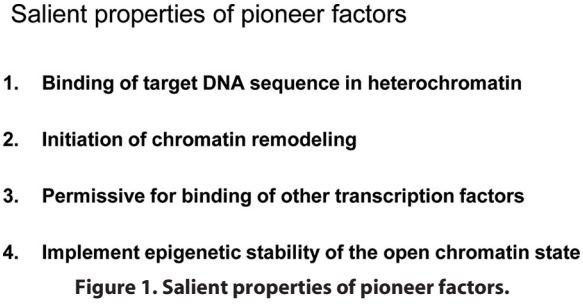

Figure 1. Salient properties of pioneer factors.

switch from "closed" naive chromatin to a state of accessibility. The label "pioneer" should thus be reserved for factors and actions shown to elicit chromatin opening from a state of complete absence of accessibility marks to the presence of such marks. On the genome scale, it is thus very important to separate the targets of pioneer action from those where the same pioneer factors only exert classical transcriptional activity at already accessible regulatory sequences; this requires an assessment of chromatin status before and after pioneer factor action in an experimental system dependent on cells that have never been exposed (in their developmental history) to the pioneer if epigenetic memory is indeed a pioneer property. Failing that, a pioneer activity may be inferred, but formal demonstration requires the before and after comparison.

Pioneers set the stage: Assisted-loading and settler factors

Pioneers appear to share the property of interacting with other TFs as do most TFs. Although very important from the biological perspective, this property is not a defining feature of pioneers. For example, the pioneer FoxA interacts with nuclear receptors such as glucocorticoid (GR/Nr3c1), estrogen (ER/Nr3a1), or androgen (AR/Nr3c4) receptors, and this allows recruitment of these nuclear receptors at subsets of enhancers (11–14) that establish hormone-responsive gene regulatory networks (15). In this context, FoxA pioneers the opening of subsets of enhancers targeted by the hormone-responsive receptors. This subsequent binding of nuclear receptors has been labeled as "assisted loading" (16), and the factors that require the open chromatin state were labeled as "settler factors" (17). The binding of a settler factor may be essential in the biological context, but it does not constitute the core pioneer activity that is restricted to initiation of chromatin opening. However, for this specific example, it appears that the interaction between FoxA and nuclear receptor may be reciprocal as nuclear receptors can also recruit FoxA to specific subsets of enhancers (18).

Pluripotency factors

The reprogramming of diverse cells such as fibroblasts into induced pluripotent stem cells(iPS) revealed the unique ability of a group of factors to reverse the differentiation process (19) toward a pluripotent state. These pluripotency factors (OSK for Oct4, Sox2, and Klf4) initiate the remodeling (opening) of both enhancers and transcription start sites (TSS). This was revealed by deposition of H3K4me1/2 at targeted enhancers and of H3K4me2/3 at TSS (20). The initial binding of these factors occurs widely at unmarked ("closed") chromatin to initiate their remodeling; they thus act as pioneer factors (21). The initial binding of OSK factors is followed by a lengthy period (weeks) of iPS cell selection that leads to the remodeling of large chromatin domains of the epigenome from a somatic to a pluripotent state. Interestingly, it was also reported that some of the iPS cell-reprogrammed enhancers require the expression and binding of more than one of the OSK factors. This suggests that pioneers may also require a cooperative action to remodel the epigenome (22). This process opens new sites for OSK binding together with sites for the accessory factor c-Myc (21). There are broad domains where OSK factors cannot bind early in reprogramming but only in the iPS cell state. These domains have high levels of H3K9me3, and this may thus constitute a barrier that contributes to refractoriness to OSK binding. Indeed, knockdown of the histone methyltransferases SUV39H1/H2 that are responsible for H3K9me3 deposition allows binding at previously inaccessible sites (20, 21, 23). The pluripotency factors Oct4 and Sox2 have critical roles in normal development to activate the zygote genome (24). In *Drosophila*, the factor Zelda has a similar role for induction of the zygote genome (25), and this is achieved through a pioneer mechanism of chromatin opening (26).

Lineage-specifying pioneer factors

The first indication that FoxA factors have pioneer activity came from showing that liver-specific FoxA-binding sites are occupied in the endoderm before liver specification (27). FoxA was then shown to bind nucleosomal DNA (28) and to open compacted chromatin (29). Genome-wide studies then showed its chromatin-remodeling activity (4, 30, 31) as well as the associated nucleosome depletion (32). In *Caenorhabditis elegans*, the FoxA-related factor PHA-4 is also critical for foregut development, and this is achieved through pioneer action (33). Interestingly, the pioneer action of PHA-4 is mostly exerted over promoter regions, and this leads to recruitment of RNA polymerase II (34). This recruitment initially leads to a poised state where RNA polymerase is paused on the promoter early on, and transcription only occurs later in foregut development.

GATA4 is also present at liver-specific enhancers in early endoderm, but its binding appeared supported by FoxA and did not show as strong an ability to bind nucleosomal DNA (29). GATA4 together with GATA6 are required for early liver development (35–37). Hence, GATA factors appear to have pioneer properties, although it may not be as effective as FoxA. Nonetheless, GATA4, like FoxA, can induce trans-differentiation into hepatocytes (38, 39).

Specification of the lymphoid, in particular macrophage, lineages depends on the factors PU.1 and C/EBPα. PU.1 is critical for development of these lineages (40). It initiates chromatin remodeling and is associated with deposition of active enhancer marks (41). Indeed, PU.1 increases chromatin accessibility and promotes nucleosome depletion (42). C/EBPα can also trigger trans-differentiation into that lineage and its binding to macrophage enhancers during that process is associated with deposition of the active enhancer marks H3K4me1 and H3K27ac. Thus, C/EBPα and PU.1 independently act as pioneer for the other during macrophage differentiation (43).

Table 1
List of transcription factors that share at least some features of pioneers
For each feature listed at the top of the table are references within the table that provide supporting evidence.

Factor	Binding to heterochromatin	Chromatin activation	Epigenetic memory: DNA demethylation	Cell fate reprogramming	Nucleosome binding	Mitotic bookmarking
Ascl1/Mash1	102	102		102, 103		
C/EBPα		43		104		
Ebf1	47, 48	47, 48	48			
Esrrb						105
Foxa	3	3, 4, 28, 31, 32	4, 69, 85	38, 39	28	77
Gata	59	59		38, 39	29	76
GR/AR	18	18				
Klf4	21, 22	21, 22		106, 107	53	
Neurod1				50, 70		
Nrf1	70	70	Inhibitory (70)			
Oct4	21, 22	21, 22		106, 107	53	
p53	100, 101	100, 101				
Pax7	44, 45	44, 45, 61	45	44		
PU.I	41, 42	41, 42		104		
Sox2	21, 22	21, 22		106, 107	53	78, 79

The pituitary intermediate lobe is specified to a unique developmental fate by the pioneer factor Pax7 (44). This is achieved through binding and chromatin remodeling of a subset of *de novo* active melanotrope-specific enhancers. The opening of these enhancers allows for recruitment of the differentiation determination factor Tpit that achieves terminal differentiation of this lineage. Pax7 pioneering results in appearance of DNA accessibility together with deposition of active enhancer histone marks (45).

Establishment of the B cell lineage requires the transcription factor EBF1 (46), and this was associated with chromatin remodeling and increased enhancer H3K4me2 (47). Some EBF1 pioneer actions were shown to depend on an EBF1 C-terminal domain that is required to trigger DNA accessibility and deposition of active chromatin marks at a specific subset of enhancers. Both C-terminally dependent and independent pioneer sites were enriched for the same EBF1 motif suggesting that the EBF1 DNA-binding site is not the defining factor between dependence and independence on the C-terminal domain. This supports a model where different pioneer interacting proteins may define functionally distinct subsets of pioneered enhancers (48).

Two neurogenic basic helix-loop-helix transcription factors shown to reprogram fibroblast to the neuronal fate appear to have pioneer activity. Indeed, Ascl1 is the driver of neuronal differentiation in association with Brn2 and Myt11, and its recruitment was associated with increased DNA accessibility (FAIRE) and with increased active chromatin marks H3K4me1 and H3K27ac together with decreases in the repressive mark H3K9me3 (49). The neurogenic factor NeuroD1 was also shown to induce similar chromatin changes at enhancers and promoters during neuronal reprogramming (50).

Pioneer interactions with DNA and chromatin

The pioneer factor activity was inferred from *in vitro* and *in vivo* footprinting experiments that showed FoxA and GATA site co-occupancy prior to hepatic specification (51). Flanking TF sites were only occupied once cells are specified toward liver identity, suggesting that pioneer factors have the unique ability to bind "closed" or naive chromatin (21, 44). Despite many genome-wide studies, the nature of this naive or closed chromatin remains vague, and the ability of pioneers to bind specific chromatin states is still defined by the negative, *i.e.* the absence of recognizable chromatin marks, and in some cases the presence of methylated cytosines in DNA. Indeed, as discussed below, some pioneers can bind methylated target DNA, whereas others appear to be methylation-sensitive.

Pioneer factors tend to have higher residency time or chromatin mobility than other TFs (18, 52) suggesting that stable chromatin–pioneer interactions may be critical for pioneer function. These stable chromatin–pioneer interactions may be explained by direct nucleosome binding, as shown for FoxA and the OSK pluripotency factors (29, 53). For FoxA, nucleosomal interaction may partly rely on a FoxA domain that resembles a linker histone H1 structure (31, 54). For the OSK factors, their ability to target partial consensus motifs may allow their DNA-binding domains to interact directly with nucleosomes (53).

In one instance, target DNA motif preference may play a role in binding stability; indeed, the pioneer Pax7 preferentially recognizes a composite motif composed of binding sites for its two DNA-binding domains, the homeodomain and paired domains, leading to greater binding stability and possibly allowing for pioneer action (44).

Epigenetic remodeling by pioneer factors

Pioneer factors provide competency for gene expression, but their binding to closed chromatin is not in itself sufficient. Indeed, chromatin remodeling is required to allow nonpioneer TF binding and transcriptional activation at newly competent regulatory sequences, primarily enhancers. The remodeling or activation of regulatory sequences from a naive or "closed" chromatin state appears to be a stepwise process (Fig. 2). None of the pioneers characterized so far have unrestricted access to the genome in heterochromatin; this was shown for FoxA, Pax7, and the pluripotency factors. This aspect is discussed below.

The first step in pioneer action is the initial binding (Fig. 2*C*) to permissive heterochromatin (Fig. 2*B*), and it appears to be rapid (*e.g.* less than 30 min for Pax7 (45)). This is followed by a phase of binding stabilization (within 24 h for Pax7) that may or may not be paralleled by nucleosomal changes that increase accessibility (31) and to the appearance of low levels of the H3K4me1 mark in the center of target enhancers (Fig. 2*D*). These "accessible" or "primed" enhancers can undergo the final

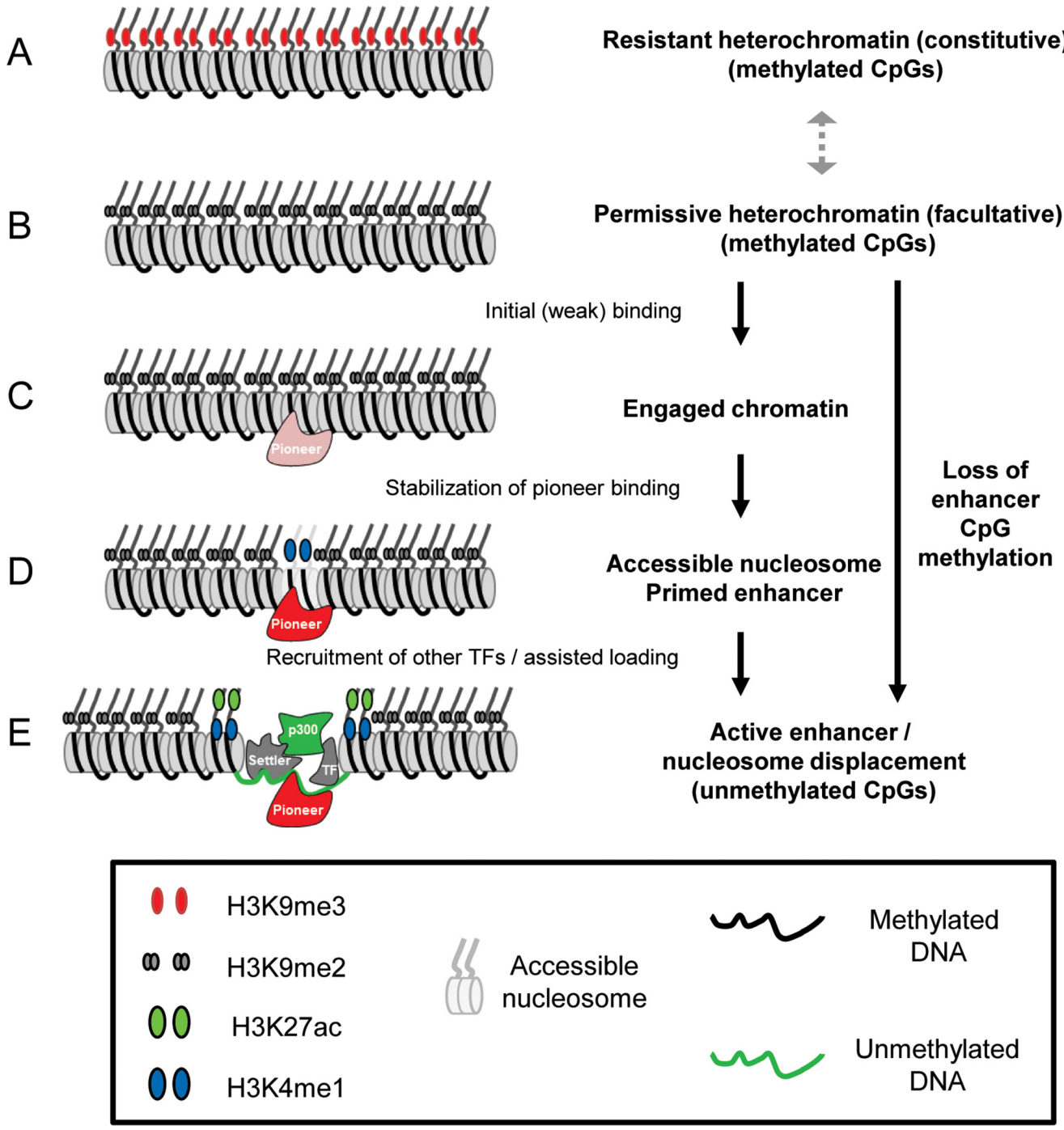

Figure 2. Current scheme of pioneer action. The permissive chromatin state for pioneer action appears to be facultative heterochromatin. Following initial weak binding of the pioneer, target site chromatin (mostly characterized at enhancers) undergoes a first transition where a central nucleosome becomes more accessible, and this may (or not) overlap with a state of Primed enhancer characterized by a weak H3K4me1 signal. Complete activation of enhancers is characterized by nucleosome depletion, bimodal distribution of H3K4me1 and H3K27ac, together with recruitment of the general coactivator p300 and other transcription factors. Whereas the ability to bind methylated DNA target sites is not a unique feature of all pioneers, for most pioneers the current evidence correlates pioneer-dependent chromatin remodeling with loss of CpG methylation at the newly accessible DNA/enhancers.

step of enhancer activation that involves the binding of other nonpioneer TFs, nucleosome depletion, and deposition of the active enhancer mark H3K27ac that is associated with the histone acetylase activity of the general coactivator p300 (Fig. 2*E*).

As most TFs, pioneers interact with chromatin remodeling proteins that are found within large complexes. These complexes have been associated with the process of transcription itself and/or its activation/initiation; the same complexes or different ones may be critical for the initial act of pioneering as well as for continued transcriptional action of pioneers. The challenge is thus to find experimental systems to separate these two actions. For example, the BRG1 ATPase of the SWI/SNF complex co-occupies many sites together with Oct4 in ES cells (55), and knockdown of BRG1 affects ES cell pluripotency (56). Oct4 is required for maintenance of open chromatin at enhancers in ES cells, and its inactivation leads to loss of accessibility at

these enhancers (57). Oct4 pioneer function is thus dependent on the chromatin remodeler Brg1. Similarly, the INO80 remodeling complex co-occupies many sites in common with pluripotency factors, and its knockdown decreases chromatin accessibility at those sites (58), suggesting that the complex may increase accessibility following recruitment by the pluripotency factors. Similarly, GATA3 was shown to require BRG1 for cell reprogramming through pioneer action (59).

The Trithorax (*Drosophila*) complex (COMPASS in yeast and MLL in mammals) is involved in activation (opening) of chromatin structure (60). Pax7 was suggested to recruit the MLL1/2 complex through interaction with its component protein WDR5 (61), and FoxA1 directs H3K4me1 deposition through recruitment of MLL3 at enhancers (62). Indeed, this complex has H3K4me1 methylation activity and thus may lead to enhancer activation. It may also be implicated in pioneering as its component protein Ash2l is recruited to sites of Pax7 pioneering (45).

For transcriptional activation, chromatin accessibility is increased at both promoters and enhancers by recruitment of the variant histones H2A.Z and H3.3 that form unstable nucleosomes (63). FoxA factors (32, 64, 65) and CLOCK:BMAL1 (66) promote recruitment of H2A.Z. This likely contributes to nucleosome instability and loss, but it is not clear that this is critical for pioneer action *per se*. Indeed, FoxA-dependent nucleosome instability is not correlated with H2A.Z deposition, and in this particular case, increased nucleosome accessibility may result from displacement of the linker histone H1 (31). FoxA factors have the unique property of containing a H1 mimic region that binds nucleosomes (28).

Barriers to pioneer binding and action

Although pioneers have the unique ability to bind their target sequence within nucleosomes in contrast to many TF that cannot, this does not mean that pioneers can bind all their target sequences in the genome. Indeed, pioneers show different binding repertoires in different cell types. For example, Sox2 binds different target subsets in mouse cortex and spinal cord (67), indicating that there are additional constraints on pioneer binding. Furthermore, the pluripotency factors OSK have a large subset of targets that only become accessible in the late phase of reprogramming toward iPS (21). The OSK-binding sites within these latter binding regions initially have higher levels of the repressive histone mark H3K9me3, and knockdown of the histone-modifying enzymes SUV39H1/H2, and SETDB1 to a lesser extent was sufficient to allow early binding of Oct4 and Sox2 to these sites in fibroblasts. Thus, the mark H3K9me3 associated with constitutive heterochromatin can constitute a barrier to OSK and possibly other pioneer binding. In addition, maintenance of heterochromatin by the histone chaperone CAF-1 is important for stable somatic cell identity as its knockdown accelerates cellular re-programming by pioneer factors (68) Other pioneers such as Pax7,[3] FoxA, and GATA (69) also exhibit lineage-specific binding repertoires. It remains to be seen whether all pioneers are subject to the same barriers or whether some may have unique limitations, and hence different permissive chromatin environments.

Whereas DNA binding by some pioneers like Pax7 is insensitive to CpG methylation within their DNA-binding site (45), DNA methylation may be an impediment to binding of TFs that have some properties of pioneers. Indeed, the factor Nrf1, predicted on theoretical bases to have pioneer action (17), will trigger chromatin access (DNase sensitivity) only if its DNA-binding site is unmethylated (70). The Nrf1 DNA-binding site is very GC-rich and contains two CpG motifs; its DNA interaction may thus be more sensitive to methylation. Another factor with methylation-insensitive DNA binding may thus be required to prime target enhancers through DNA demethylation to allow Nrf1 binding and action. There may thus be a hierarchy of pioneers with differing potencies; "true" pioneers may be considered to be those with methylation-insensitive DNA binding and an ability to induce DNA demethylation, but the biological context may provide an argument to consider factors such as Nrf1 as pioneer. For example, global DNA demethylation occurs at two critical stages of mammalian development, in the pre-implantation embryo and during primordial germ cell proliferation and migration (71, 72). DNA methylation-sensitive pioneers may thus act as classical TFs in most cells but transiently behave as pioneers during development. Such limitation on pioneer action could explain specific roles played by pioneers in distinct cell types. The detailed assessment of the pioneer mode of action is thus critical to understand their role in lineage specification.

Many pioneers exhibit extensive binding site subsets of low affinity that are resistant to remodeling (45, 53). Some of these sites appear to have degenerate DNA-binding site sequences and were proposed to represent a mechanism for scanning targets. Notwithstanding this possibility, this mechanism does not provide an explanation for selection of specific pioneering sites.

Stability of pioneer-induced chromatin remodeling

During development, pioneers stably reprogram the chromatin landscape leading to a stable cell identity. As such, they would implement a memory for long-term maintenance of cell identity. During mitosis, chromatin is disassembled and reconstituted after replication. There are mechanisms to reconstitute the daughter cell chromatin landscape as in the mother cell (73). It was proposed that pioneers, and possibly other TFs, bookmark the chromatin during mitosis to allow re-establishment of active regulatory networks. Indeed, although many TFs were shown to be excluded from mitotic chromosomes (74, 75), some pioneers appear to remain bound to mitotic chromosomes; for example, GATA1 binding is maintained in mitotic chromosome at a tissue-specific subset of 5% of its chromatin targets (76). Surprisingly, this study also identified mitosis-specific binding of GATA1 at sites that do not contain the consensus GATA motif. Both specific and nonspecific binding sites on mitotic chromosomes were also observed for FoxA1 (77) where specific FoxA1 binding occurs at 15% of its interphase targets. Recently, Sox2 and Oct4 were also shown to remain bound during mitosis (78, 79). In this last study, the authors also show, using live imaging techniques, that cross-linking with formaldehyde leads to eviction of most TFs from mitotic chromo-

[3] A. Mayran and J. Drouin, unpublished data.

somes. They proposed a model where most TFs remain bound during mitosis to maintain the original program despite only showing this for the well-characterized pioneer Sox2.

The most stable epigenetic mark associated with inactive heterochromatin is DNA methylation (80). Indeed, promoters and CpG-rich promoter regions (CpG islands) that are transcriptionally active are largely demethylated, and this is required for activity. Similarly, active enhancer sequences are hypomethylated, and the patterns of enhancer hypomethylation are associated with cell-specific gene–expression programs (81). Following replication, hemi-methylated CpG dinucleotides are recognized and methylated by the Dnmt1–Uhrf1 complex (82, 83). Maintenance of DNA methylation patterns by this mechanism thus ensures stability of lineage-specific gene-expression programs. As inactive (closed) regions of chromatin that are targeted by pioneers have high DNA methylation, it is expected that pioneers should bind their target sequence independently of DNA methylation, and this is indeed the case for FoxA and Pax7, although there may be exceptions as for Nrf1 discussed above (70).

Whereas direct DNA binding by FoxA and Pax7 is not impaired by CpG methylation of their binding site, their action leads to local demethylation of flanking enhancer sequences beyond the DNA-binding site (45, 69). This demethylation is associated with epigenetic memory and maintenance of an open/accessible chromatin environment (45).

A few pioneer factors were investigated for their impact on DNA methylation. FoxA1 can induce DNA demethylation (4) thus demonstrating its impact on the DNA methylation landscape. Active DNA demethylation can be achieved by the Tet enzymes (84), but for FoxA-dependent demethylation activity, it was rather suggested to require recruitment of a FoxA1 DNA repair complex (85). Also, EBF1 and Pax7 pioneer actions lead to loss of DNA methylation (45, 48). The mechanism of pioneer-induced DNA demethylation remains uncertain as the known DNA demethylation Tet pathway could not be implicated in either FoxA or EBF1 action (48, 85). It is noteworthy that FoxA-dependent chromatin remodeling can occur independently of replication, whereas DNA demethylation is impaired by blockade of replication (69). These data clearly separate two steps in pioneer action, and these are consistent with the time frames of action defined in an inducible system for Pax7 (45).

Pioneer factors in cancer

In view of their chromatin remodeling activities, pioneer TFs have the potential for significant epigenetic alterations as seen in cancer. Indeed, FOX family genes are involved in several cancers (86). Overexpression of Foxa1 is associated with a poor prognosis in prostate cancer (87), although it is generally a good prognosis of breast cancer (88). Point mutations of FOXA1 were also found in some prostate cancers, and this was associated with decreased androgen signaling and increased tumor growth (89).

In ER^+ breast cancer cells, ER binding requires FOXA1 at many binding sites showing the role of FOXA1 in driving hormone response of these tumors (90). Similarly, AR binding is also influenced by FOXA1; indeed, some AR-binding sites are lost in cells depleted of FOXA1; however, many sites are also gained suggesting a more complex relationship of FOXA1 with AR than with ER (87, 91).

Also, FOXM1 is amplified in some breast cancers (92), in non-Hodgkin's lymphomas (93), or in malignant peripheral nerve sheath tumors (94). FOXM1 is activated through post-translational phosphorylation by ERK, and FOXM1 activation is associated with a poor prognosis for many human cancers such as lung, medulloblastoma, breast, gastric, and pancreatic cancers.

Chromosomal translocations leading to fusion of the N-terminal DNA-binding domain of PAX3 or PAX7 with the C-terminal transactivation domain of FOXO1 (FKHR) were found in rhabdomyosarcomas. These fusion proteins act as much more potent activators than the native PAX3 or PAX7 (95). PAX3–FOXO1 was shown to lead to activation of genes involved in cancer development and to inappropriate expression of developmental TFs (96). PAX7 and FOXO1 both have pioneer activity (44, 97). As such, these fusion proteins may also function as pioneers. Furthermore, *FOXO3* or *FOXO4* are trans-located to *MLL* gene in acute lymphoblastic leukemia leading to increased cell proliferation (98). FOXO proteins function as tumor suppressors (99), and their loss of activity due translocation or deletion may also lead to increased tumorigenesis.

Finally, two studies showed that the tumor suppressor p53 (TP53) can engage inaccessible chromatin. In one study, p53 binding led to deposition of H4K16ac together with H3K27ac at non-TSS sites. However, neither gain of chromatin accessibility nor deposition of H3K4me1 accompanied these changes, thus possibly defining a unique chromatin environment specific to p53 (100). A recent study showed that after DNA damage, a subset of p53-binding sites are associated with *de novo* accessibility assessed by ATACseq possibly highlighting a canonical pioneer action of p53 (101).

Perspective

As exemplified in this Minireview, the critical aspects of pioneer action are still the least understood. First and foremost, the molecular basis for pioneer access to their target DNA sequences in closed chromatin remains obscure. There may be more than one underlying mechanism as the mechanism proposed for FoxA interaction with nucleosomal DNA, namely its putative linker H1 mimicry binding interactions, does not seem to apply to other pioneers. The question remains whether all pioneers use the same molecular strategies to elicit chromatin remodeling. They may also may differ in their ability to access to various "flavors" of heterochromatin.

The initial binding and action of pioneers to closed chromatin regions and the initiation of chromatin remodeling are the critical features that distinguish pioneers from other TFs. Is there something unique about pioneer action on chromatin at this initiating event, or is the recruitment of chromatin remodeling complexes at that initiating event the same as those that occur during activation of enhancer function in transcription? This latter possibility would imply that the only unique aspect of pioneer action is the ability to recognize target sites in "closed" chromatin. Alternatively, this ability may be operating in conjunction with recruitment of a unique set of chromatin

remodelers involved in initiating chromatin opening but not necessarily involved in maintenance of this accessible state. To answer these difficult questions requires the availability of experimental systems where the specific steps of pioneer action can be followed and investigated. Is there something unique about the maintenance of chromatin accessibility at pioneer sites, or does this simply result from recruitment of enhancer machinery (combination of TFs, chromatin remodelers, and chromatin modifiers) leading ultimately to changes in the most stable epigenetic mark, demethylation of DNA cytosines?

Addressing these questions is paramount to understand pioneer action and to use this knowledge in the context of cell fate reprogramming. Understanding the nature of the cell fate reprogramming that may occur during tumorigenic processes will be critical for therapeutic development of cell therapies.

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