Pioneer factor Pax7 initiates two-step cell-cycle dependent chromatin opening

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Summary

Pioneer transcription factors direct cell differentiation by deploying new enhancer repertoires through their unique ability to target and initiate remodelling of closed chromatin. The initial steps of their action remain undefined although pioneers were shown to interact with nucleosomal target DNA and with some chromatin remodelling complexes. We now define the sequence of events that provide pioneers with their unique abilities. Chromatin condensation exerted by linker histone H1 is the first constraint on pioneer recruitment, and this establishes the initial speed of chromatin remodelling. The first step of pioneer action involves recruitment of the LSD1 H3K9me2 demethylase for removal of this repressive mark, as well as recruitment of the MLL complex for deposition of the H3K4me1 mark. Further progression of pioneer action requires passage through cell division, and this involves dissociation of pioneer targets from perinuclear lamins. Only then, the SWI/SNF remodeling complex and the coactivator p300 are recruited, leading to nucleosome displacement and enhancer activation. Thus, the unique features of pioneer actions are those occurring in the lamin-associated compartment of the nucleus. This model is consistent with much prior work that showed a dependence on cell division for establishment of new cell fates.

Introduction

Cell fates are established and maintained through the action of specific combinations of transcription factors, including cell-restricted factors that define unique cell identities. The implementation of new cell fates relies on activation of new enhancer repertoires and this is achieved by pioneer transcription factors through their unique ability to access target sites in closed chromatin and trigger chromatin opening[1]. Many aspects of this general scheme remain undefined, notably pioneer interaction with closed chromatin and the initial events of chromatin remodelling[1].

Investigation of DNA sequences targeted for pioneer action did not show preferential occurrence of sequence subsets, with some pioneers exhibiting degenerate recruitment sites[2] while others have stronger recruitment at pioneered sites[3,4]. Closed chromatin access may include the ability to bind target DNA sequences on nucleosomes, one of the earliest features ascribed to many, but not all[5], pioneer factors[6]. Structural evidence suggests that pioneer binding may weaken the interaction of DNA with nucleosomes and hence contribute to initial chromatin alteration[7-9]. This is compatible with the appearance of so-called "accessible nucleosome conformation" caused by FOXA recruitment[10] or by subtle chromatin rearrangements that precede frank chromatin opening following the action of the pioneer Pax7 (REF.[3]). However, nucleosomal organisation cannot on its own explain barriers to pioneer action, and the data rather suggest that chromatin environment dictates whether chromatin is permissive or not for pioneer action. For example, the highly compacted constitutive heterochromatin prevents pioneer access[11,12]. The nature of crucial events initiating pioneer action remain elusive[1].

It is likely that chromatin remodelling complexes identified for their role in development or in transcription are also involved in chromatin opening by pioneer factors. For example, the MLL complex is recruited to FOXA-pioneered sites that are targets of the oestrogen receptor in breast cancer cells[13]. Another pioneer, Pax7, interacts with the methyltransferase CARM1 and the MLL1/2 proteins[14]. The pioneers OCT4, GATA3 and ISL1, interact with Brg1, the ATPase of the SWI/SNF chromatin-remodelling complex[15-17]. But it is presently unclear how and when these interactions may be relevant to pioneer-dependent chromatin opening.

In the present study, we used an inducible system to define the time course of chromatin opening at Pax7-pioneered enhancers[3]. Amongst its different roles[18], Pax7 specifies the intermediate pituitary melanotrope cell fate by opening a repertoire of >2000 enhancers[3,19]. While Pax7 is necessary to trigger chromatin opening, it also requires cooperation with the nonpioneer transcription factor Tpit to fully open and activate enhancer chromatin[20]. We now show that Pax7

pioneer action occurs in two steps, a first step that is limited by Pax7 recruitment strength and inversely correlated with the level of linker histone H1. And a second step that requires passage through replication and cell division, for recruitment of the SWI/SNF complex, nucleosome displacement and enhancer activation. This sequential scheme of pioneer action defines a conceptual framework to further probe and control the pioneering process.

Results

Pax7 primes enhancers for activation

We previously provided an exhaustive analysis of chromatin opening by the pioneer factor Pax7 at two enhancer repertoires, one where Pax7 action results in complete enhancer activation and another where enhancers only reach the primed state[3]. To study the kinetics of enhancer opening by Pax7, we used a tamoxifen-inducible ER-Pax7 chimera system (Fig. 1a). The present study compared subsets of enhancers that are either activated or primed in response to Pax7 with a set of constitutively active enhancers where Pax7 binding does not alter chromatin organization (Fig. 1b and Supplementary Fig. 1a, b). Having previously observed rapid Pax7 binding to pioneered sites in closed chromatin but relatively slower remodelling[3], we first assessed whether the primed state is indeed a transitory state towards complete enhancer activation. The status of pioneered enhancers was assessed by ChIP-Seq for the chromatin mark histone monomethylated histone H3 Lys4 (H3K4me1) at different times following activation of ER-Pax7 (Fig. 1c). Thus, pioneered enhancers are observed first (12h) in the primed state followed by complete activation (Fig. 1c). The primed state was previously documented as presenting with a weak single peak of H3K4me1, whereas fully activated enhancers present with a stronger bimodal distribution of H3K4me1 that reflects nucleosome displacement at the center of pioneered enhancers[1].

Pioneered sites are heterogeneous

ChIP-Seq analyses of pioneered sites show significant heterogeneity with regards to Pax7 recruitment and deposition of chromatin marks (Fig. 1b). To assess the variability in implementation of Pax7 chromatin opening, we selected a panel of 14 pioneered sites representative of the Pax7 recruitment distribution (Fig. 1d). We validated that pioneered enhancers are first remodelled into a primed state by H3K4me1 ChIP-qPCR using two sets of primers, one centered on the Pax7 binding site and another shifted to one of the H3K4me1 bimodal deposition summit: indeed, H3K4me1 central deposition (average half-time of 24h) occurs before lateral (average half-time of 32h) accumulation (Fig. 1e,f). Prior data had suggested interaction between Pax7 and the MLL complex that has H3K4me1 methyltransferase activity[14]. We therefore assessed MLL complex recruitment at Pax7 pioneered enhancers by ChIP-qPCR for the MLL3/4 and Ash2 components of the MLL complex: both MLL4 and Ash2 are recruited with a half-time (average of the 14 sites) of ~12 hours following Pax7 activation (Fig. 1f and Supplementary Fig. 1c). This half-time corresponds to a time when enhancers are in a primed state (Fig. 1c) and precedes bulk H3K4me1 deposition (Fig.1f). This delay taken together with the prior demonstration of a delay between chromatin opening measured by ATAC-Seq and Pax7 recruitment[3] suggests that chromatin opening initiated by Pax7 may involve sequential steps.

Chromatin opening occurs in two steps

To assess the sequence of events leading to chromatin opening, we performed ChIP-qPCR time courses for various chromatin state markers and chromatin remodellers (Fig. 2a-d). Pax7 recruitment itself has an average half-time of ~12 hours and we found a similar time course for recruitment of Tpit, a cooperating transcription factor that is required for chromatin opening at the Pax7 pioneered enhancers[20] (Fig. 2a). Recruitment of these factors parallels MLL4 and Ash2 recruitment: they all precede deposition of H3K4me1 at the center of enhancers (Fig. 2a). We next assessed chromatin opening by ATAC-qPCR: the half-time of chromatin opening of ~24 hours is similar to H3K4me1 deposition (Fig. 2b). These longer time courses are similar to those

of nucleosome displacement at these enhancers as revealed by ChIP-qPCR for histone H3 and for recruitment of Brg1 (Fig. 2b). The recruitment of the SWI/SFN complex (Brg1) occurs in parallel with recruitment of the general coactivator and histone acetylase p300 and of the cohesin complex protein SMC1 (Fig. 2b). These precede deposition of the active enhancer mark H3K27ac (Fig. 2c). These time courses clearly identify two steps in Pax7-dependent chromatin opening: an initial step marked by Pax7 recruitment and a delayed second step that involves nucleosome displacement (Fig. 2a-c and Supplementary Fig. 2a, b).

We previously showed depletion of the repressive histone mark H3K9me2 that is typical of facultative heterochromatin at the Pax7 pioneered enhancers[3]. We next assessed the time course of this depletion and observed a half-time of ~12 hours (Fig. 2d). Since facultative heterochromatin is compacted, we assessed the levels of linker histone H1 at these sites and observed a time course of H1 depletion similar to that of H3K9me2 depletion (Fig. 2d). The initiation of Pax7 pioneering thus correlates with an initial perturbation of the chromatin environment represented by the loss of linker histone H1 and the loss of the facultative heterochromatin mark H3K9me2, together with implementation of the primed state reflected by a weak deposition of H3K4me1 (Fig. 1c).

Pioneering kinetics depend on Pax7 recruitment strength

While the average time response curves defined two steps in the pioneering process, examination of parameters for the individual 14 pioneer sites revealed heterogeneity in the kinetics of pioneer action (Fig. 2e). Color-coded individual time course curves for Pax7 recruitment, histone H3 depletion, ATAC signal, H3K4me1 deposition, Brg1 recruitment and H3 depletion indicate that the stronger Pax7 recruitment sites (Fig. 1d, red) are remodelled quicker than the weaker sites (blue). The correlation between recruitment strength and half-time of remodelling is observed for

all parameters (Fig. 2f). Similar correlations are observed for all pioneered enhancers genome-wide (Supplementary Fig. 3a). Computation of time course curves for the five strongest and five weakest sites indicates an average delay of ~6 hours between the two subgroups (Supplementary Fig. 3b). These data clearly support the conclusion that recruitment strength determines the onset of chromatin remodelling at pioneered sites.

To assess whether these correlations relate to long-term or initial levels of Pax7 recruitment, we performed similar comparisons at 1 hour after Pax7 activation. These revealed that recruitment strength at 1 and 48 hours are directly correlated (Fig. 2g and Supplementary Fig. 4) and that initial recruitment is inversely correlated with the time course of chromatin remodelling (Fig. 2h).

We next assessed the impact of initial chromatin organization on the strength of Pax7 recruitment. Whereas nucleosome content reflected by total histone H3 ChIP levels and the level of the repressive mark H3K9me2 (Supplementary Fig. 5a) do not correlate with Pax7 recruitment, chromatin compaction dependent on linker histone H1 determines the initial (1h) strength of Pax7 recruitment (Fig. 2i). Similar observations were made at 48h after Pax7 activation (Supplementary Fig. 5b) and confirmed genome-wide by UMAP representation of Pax7 recruitment compared to histone H1 levels (Fig. 2j). Hence, the higher the level of histone H1, the weaker the initial Pax7 recruitment and the longer it takes to remodel these sites (Fig. 2i). In summary, chromatin condensation reflected by the level of histone H1 constrains Pax7 recruitment and determines the time course of chromatin remodelling.

Activation of Pax7 primed enhancers requires cell division

The half-time of the second step of the pioneering process (~24 hours) corresponds roughly to the half-time for AtT-20 cell division. Thus, the second step of the pioneering process may require DNA replication or passage through cell division. We assessed this by blocking AtT-20 cell replication for 12 hours with mimosine (Fig. 3a, b). Mimosine-blocked AtT-20 cells are viable and

168 can re-enter cell cycle upon release (Supplementary Fig. 6a). We assessed each step of the
169 pioneering process by comparison of mimosine-blocked with normal cycling cells. These
170 experiments indicate that Pax7 recruitment and H3K9me2 depletion, two parameters of the first
171 pioneering step, occur independently of cell replication and division (Fig. 3c). In marked contrast,
172 all marks of the second step of the pioneering process are blocked by mimosine treatment,
173 including chromatin opening assessed by ATAC-qPCR, Brg1 and p300 recruitment, and
174 deposition of H3K27ac (Fig. 3d and Supplementary Fig. 6b). Since prior experiments showed that
175 the first step of the pioneering process yields enhancers in the primed state, we assessed the
176 pattern of H3K4me1 in mimosine-blocked cells that fail to undergo the second step of the
177 pioneering process. The ChIP-Seq average profiles of pioneered enhancers in mimosine-blocked
178 cells remain in the primed state (Fig. 3e) in contrast to normal cycling cells that are in the activated
179 state at a similar time following Pax7 activation (Fig. 1c).

180 **Cell division is required for dissociation from nuclear lamins**

181 Since the first change observed in parallel with Pax7 recruitment is the depletion of the repressive
182 mark H3K9me2, we queried a putative mechanism for this depletion. The analysis of proteins
183 associated with Pax7 in RIME experiments[21] provided two candidate demethylases, KDM1A
184 (LSD1) and KDM3A (Harris et al. In preparation). Hence, we assessed recruitment of these
185 demethylases at Pax7 pioneered enhancers and found that KDM1A is recruited with a half-time
186 corresponding to step 1 of the process (Fig. 4a) whereas KDM3A is recruited at step 2 of the
187 process (Supplementary Fig. 7a). The early loss of H3K9me2 may be a mechanism to alter the
188 localization of pioneered sites in the nucleoplasm. Indeed, H3K9me2-marked loci are found in the
189 nuclear periphery B compartment and their localization is altered by removal of H3K9me2
190 (REF.[22]). We thus assessed the association of the Pax7 pioneered sites with lamin B1, a marker
191 of the nuclear B compartment (Fig. 4b) and found that high levels of lamin B1 correlated with high
192 levels of H3K9me2 at pioneered sites (Fig. 4c). Despite the loss of H3K9me2 at step 1, we

observed dissociation of pioneered sites from lamin B with a half-time corresponding to the cell cycle-dependent second step (Fig. 4a). If the requirement for cell division observed for step two of the pioneering process depends on nuclear compartment switching, the blockade of cell division should prevent dissociation from lamin B, and this is indeed what was observed in mimosine-blocked cells (Fig. 4d). In this context, we would expect that enhancers that are de novo primed by Pax7 would not show significant H3K9me2 depletion as is indeed observed (Fig. 4e). We surmise that the slight decrease of H3K9me2 at primed sites is not sufficient for compartment switching and this is supported by the similar levels of H3K9me2 at sites that are were already in the primed state before Pax7 activation and that get transcriptionally activated by Pax7 (Activated enhancers, Fig. 4e). Further, we observe recruitment of the SWI/SNF complex only after full activation but not with the primed state as revealed by Brg1 ChIP-Seq (Fig. 4e), in agreement with restricted nuclear localisation of Brg1 in the central nucleoplasm[23]. We found similar features for recruitment of RNA Pol II and components (MED1 and MED12) of the Mediator complex (Supplementary Fig. 7b). Why do some enhancers fail to complete the process of activation? A failure to recruit an essential cooperating transcription factor could be responsible and indeed, we observe no recruitment of Tpit, a nonpionner factor required for full activation of melanotrope enhancers[20], at de novo primed sites (Fig. 4e).

In conclusion, and taken collectively, the present data suggest a model for pioneer factor action (Fig. 4f, g) in which the first steps are compatible with actions within the perinuclear compartment B: these include removal of repressor marks together with deposition of activation marks corresponding to the primed state. The ensuing steps for enhancer opening require passage through cell division that allows for lamin dissociation, and presumably switching to the A compartment, where the loci become accessible to the SWI/SNF complex for nucleosome displacement and transcriptional activation.

Discussion

The present work shows that chromatin compaction elicited by histone H1 determines the strength of pioneer Pax7 recruitment and speed of its actions. Beyond this limiting step, the different parameters of chromatin opening follow a similar time course while the next limiting step is imposed by cell division and its importance for lamin, and presumably compartment B, dissociation (Fig. 4g). The association of SWI/SNF (Brg1) recruitment and nucleosome displacement is consistent with the nuclear sublocalization of BRG1 and Brm in compartment A, but not in the perinuclear compartment B, of the nucleoplasm[23].

As the ability of loci to switch from compartments B to A depends on the level of H3K9me2 (REF.[22] [24]), it follows that enhancers that are primed (but not activated) by Pax7 do not show significant H3K9me2 depletion (Fig. 4e). In contrast, these primed enhancers have deposition of H3K4me1 presenting as a single weak peak (Fig. 4f). Consistent with an early recruitment of the MLL complex (Fig. 2a), the data indicate that targeted enhancers can be primed in the lamin-associated compartment including deposition of H3K4me1. The limiting factor for complete enhancer activation is thus controlled by demethylation of H3K9me2 and this appears to require the cooperating nonpioneer Tpit (Fig. 4e). The variable effectiveness of Pax7 to open chromatin at different sites may thus be related to chromatin constraints such as dictated by histone H1, and/or to the relative affinity for DNA binding sites and/or to nonpioneer interaction. In models that may involve different cooperating nonpioneers, a single pioneer factor may thus implement various subsets of primed enhancers ready for activation in response to an array of developmental or signaling cues mediated through the activities of different nonpioneers.

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AUTHOR CONTRIBUTIONS

A.G. and J.D. conceived the study, A.G., A.B. and J.D. conceived and designed the experiments, A.G., J.D.-G. and V.L.-R. performed ChIP experiments, A.G. and J.D. wrote the manuscript.

COMPETING INTERESTS

The authors declare no competing financial interests.

Legends to Figures

Fig. 1 | Pax7 primes enhancers for activation. a) The role of Pax7 in specification of the intermediate pituitary melanotrope cell fate is illustrated in contrast to the corticotrope cell fate. These two hormone-secreting lineages express the same hormone precursor gene, proopiomelanocortin (POMC), and their fates are determined by the Tbox transcription factor Tpit. The mouse AtT-20 cells are a model of the corticotrope lineage and Pax7 expression reprograms these cells towards a melanotrope-like fate. **b**) Heat map representation of ChIP-Seq and ATAC-Seq data for three subsets of Pax7-targeted enhancers. Data are shown before (0h) and after 48h induction of Pax7 activity in AtT-20 cells harbouring a tamoxifen-inducible ER-Pax7 chimera[3]. Pioneered enhancers have no marks before Pax7 activation and present with hallmarks of transcriptionally active enhancers after, namely appearance of an ATAC-Seq signal together with a bimodal distribution of H3K4me1 deposition. Pax7 primed enhancers also show appearance of weaker ATAC-Seq and H3K4me1 signals, the latter presenting as a single weak peak. Finally, a set of constitutively active enhancers serve as control for comparisons. These enhancers bind Pax7, but this binding is not associated with any change in chromatin signature. These enhancer subsets were previously described[3,4]. **c**) Average profiles of H3K4me1 deposition at subsets of Pax7-remodelled enhancers at indicated times following tamoxifen (Tam) activation of ER-Pax7. **d**) Selection of 14 Pax7-pioneered sites used for detailed investigation by qPCR and color-coded from red to blue according to strength of Pax7 recruitment. Pax7 and H3K4me1 ChIP-Seq, and ATAC-Seq, profiles are shown for these individual peaks at 48h following Pax7 activation. **e**) Time course of H3K4me1 deposition determined by ChIP-qPCR at the 14 pioneered sites illustrated above. For each site, a red curve illustrates H3K4me1 accumulation under the Pax7 peak (centered), whereas a blue curve represents H3K4me1 deposition over a lateral peak of deposition (shifted) that characterizes the bimodal H3K4me1 distribution of fully active enhancers. **f**) Average (of 14 pioneered sites) time course of H3K4me1 accumulation (centered and shifted)

is compared with ChIP-qPCR data for recruitment of two protein components of the MLL remodelling complex, MLL4 and Ash2. Data represent the means ± sem of duplicate biological samples.

Fig. 2 | Pax7-dependent chromatin opening occurs in two steps. a) Average (14 pioneered sites of Fig.1d represented as means ± sem of duplicate biological samples, n ≥ 2) profiles of ChIP-qPCR data for Pax7, Tpit, MLL4 and Ash2 that are recruited with a similar time course (T ½ ~ 12 hrs) compared to accumulation of H3K4me1 (center). **b**) Compared to Pax7 recruitment, ATAC-Seq signals and recruitment of Brg1, p300 and SMC1, together with depletion of Histone H3 occur with an average T ½ of ~ 24h (n ≥ 2). **c**) Deposition of activation mark H3K27ac and bimodal H3K4me1 (shifted) are slightly delayed compared to ATAC signal (n ≥ 2). **d**) Depletion of the repressive mark H3K9me2 and of linker Histone H1 occur with a T ½ of ~ 12h (n ≥ 2). **e**) Individual site ChIP-qPCR time course data for recruitment of Pax7, H3K4me1 and Brg1, together with time courses of ATAC signals and depletion histones H1 and H3 shown for the 14 pioneered sites color-coded (in Fig.1d) from red to blue to reflect the strength of Pax7 recruitment. **f**) Correlations between Pax7 recruitment strength and various markers depicted in (e). **g**) Correlation between rapid (1h) and long-term (48h) Pax7 recruitment strength. **h**) Inverse correlation between initial (1h) Pax7 recruitment strength and the average half-times of all markers of chromatin remodelling at the 14 pioneered sites. **i**) Correlations between the initial levels of histone H1 at the 14 pioneered sites and the strength of Pax7 recruitment and with the half-time of chromatin remodelling markers. **j**) UMAP representation of Pax7 recruitment strengths at the indicated times after Tam addition compared to initial histone H1 levels measured by ChIP-Seq at pioneered enhancers depicted in Fig. 1b.

Fig. 3 | Activation of Pax7 pioneered enhancers requires cell division. a) Experimental design for assessment of chromatin remodelling in cells with mimosine-blocked cell cycle compared to normally cycling cells. **b**) FACS profiles of normal (no mimosine) cells compared to cells incubated for 12h with mimosine that exhibit cell-cycle arrest in G1. Representative of n=2 is shown. **c**) Both recruitment of Pax7 and depletion of H3K9me2 measured by ChIP-qPCR at the 14 pioneered sites are not affected by blocking AtT-20 cell cycle with mimosine (n=2). **d**) In contrast, chromatin opening (ATAC) recruitments of Brg1, p300 and H3K27ac measured by ChIP-qPCR are all severely curtailed in mimosine-blocked cells (n=2). **e**) Average profiles of H3K4me1 accumulation at Pax7-remodelled enhancer subsets measured by ChIP-Seq at different times after Tam activation of ER-Pax7.

Fig. 4 | Pax7-dependent chromatin remodelling involves H3K9me2 demethylation and dissociation from lamins. a) Average (14 pioneered sites of Fig.1d represented as means ± sem of duplicate biological samples) time courses for depletion of H3K9me2 and dissociation from lamin B1 measured by ChIP-qPCR compared to the time courses of recruitment for the H3K9me2 demethylase, KDM1A (LSD1). **b**) Pax7 pioneered sites have high levels of H3K9me2 and lamin B1 before Pax7 action. **c**) Correlation between initial levels of H3K9me2 and lamin B1 at 14 pioneered sites before Pax7 action ($n \geq 2$). **d**) Lamin depletion is prevented by cell cycle blockade with mimosine (n=2). **e**) Heatmaps of ChIP-Seq profiles for Pax7, H3K4me1, H3K9me2, Tpit and Brg1 at subsets of Pax7 remodelled enhancers. Data for other markers presented in Supplementary Fig. 7. **f**) Schematic representation of proteins shown in the present work to be associated with either primed or active enhancer states. **g**) Sequential model of Pax7 pioneer action. Pax7 pioneer targets are enriched in H3K9me2 and associated with lamin B1, which is typical of chromatin that is present in the perinuclear B compartment. Step 1 of the pioneering process involves recruitment of the H3K9me2 demethylase KDM1A (LSD1), together with the MLL complex. These lead to demethylation of H3K9me2 and H3K4me1 deposition, respectively.

Cell cycle blockade with mimosine prevents Pax7-dependent remodelling from proceeding further. At mitosis, masking of the H3K9me2 mark by H3S10 phosphorylation accompanies dissociation of A and B compartments of the nucleus[22]. Following mitosis and nuclear reassembly, Pax7-pioneered targets that have undergone H3K9me2 demethylation will remain in the central nuclear A compartment where the Brg1 and Brm ATPase's of the SWI/SNF complex are localised[23] and recruited for nucleosome displacement and complete enhancer activation. Pax7 primed enhancers do not recruit the cooperating nonpioneer factor Tpit and hence, the cooperation between Tpit and Pax7 appears required for significant H3K9me2 demethylation and continuation of the Pax7-dependent remodelling process to the cell cycle-dependent step 2.

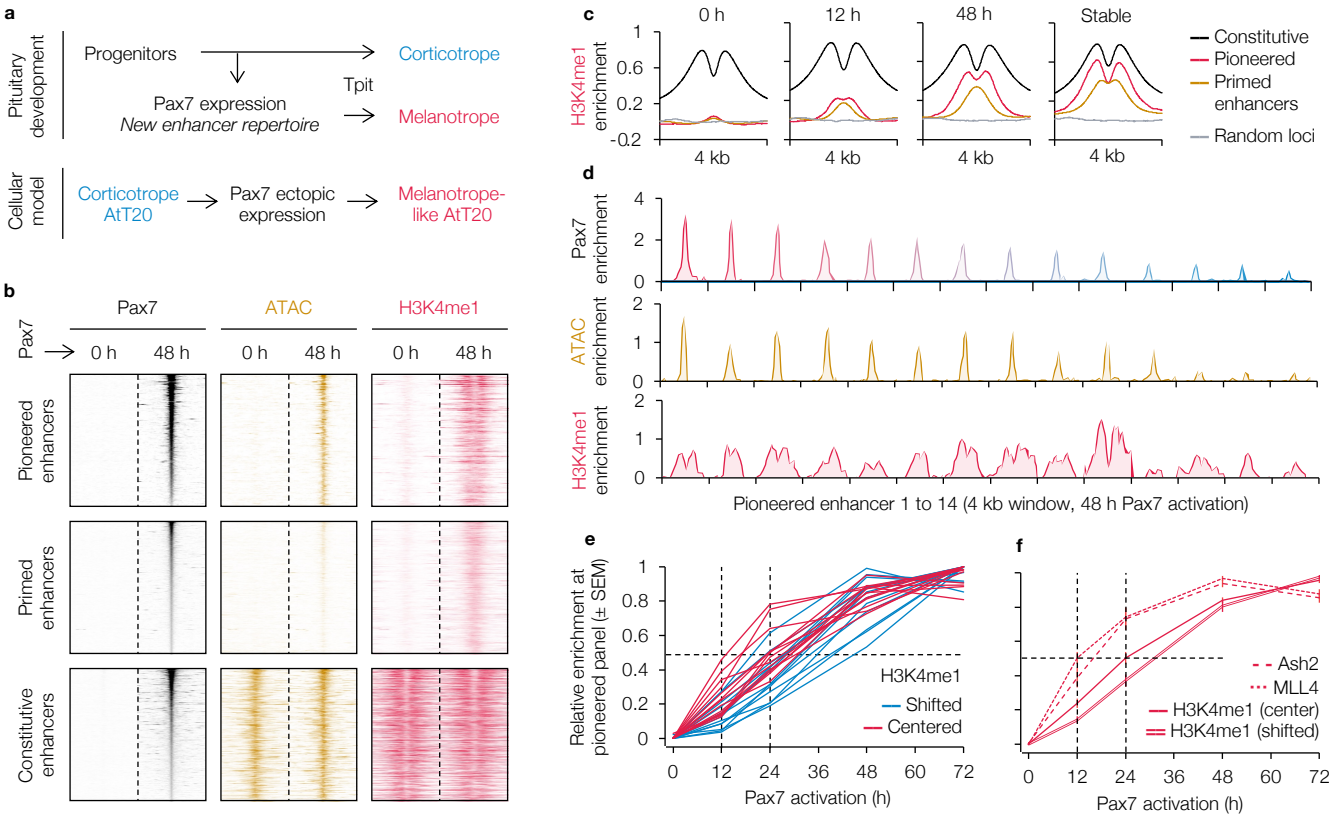

Fig. 1 | Pax7 primes enhancers for activation.

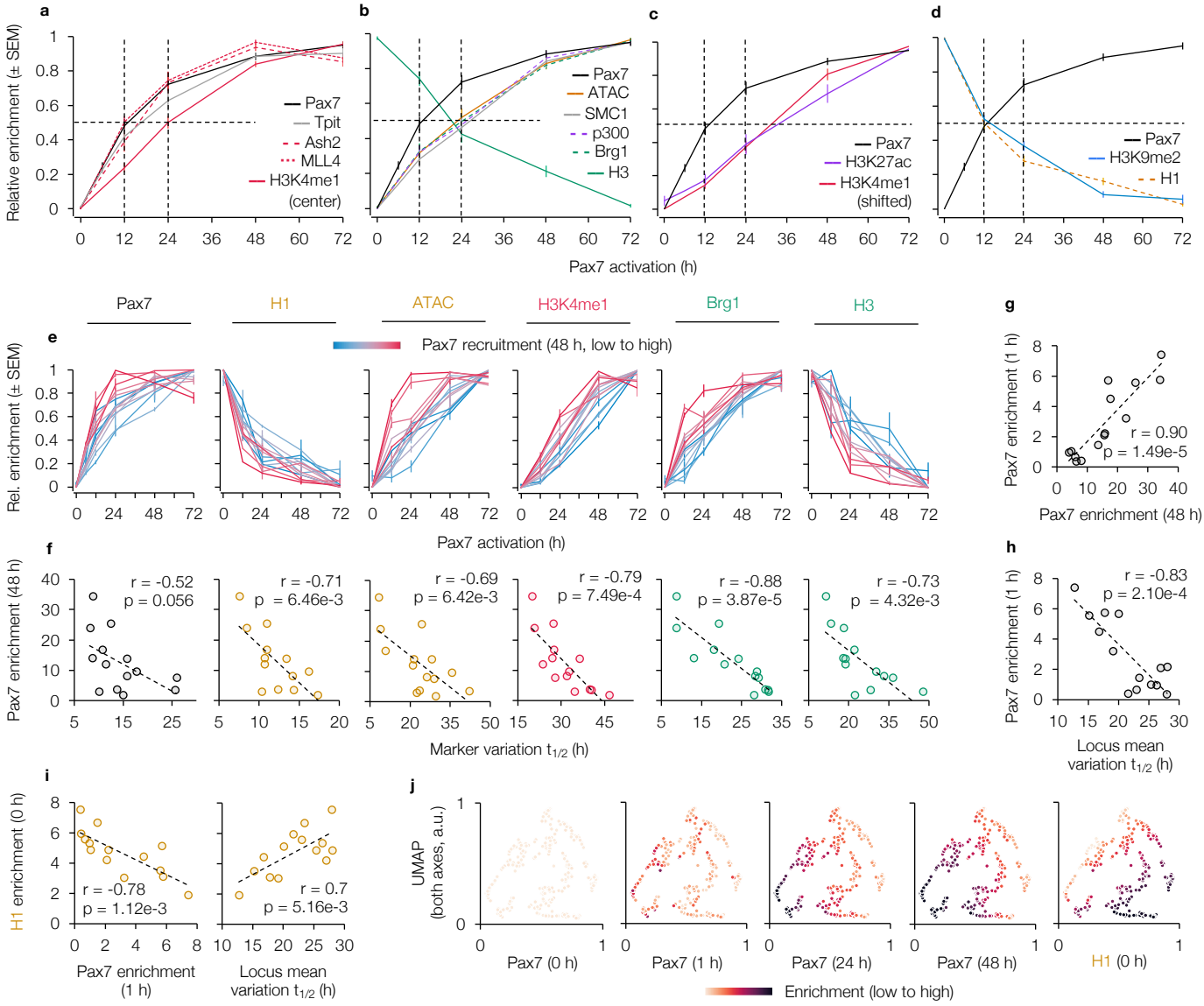

Fig. 2 | Pax7-dependent chromatin opening occurs in two steps.

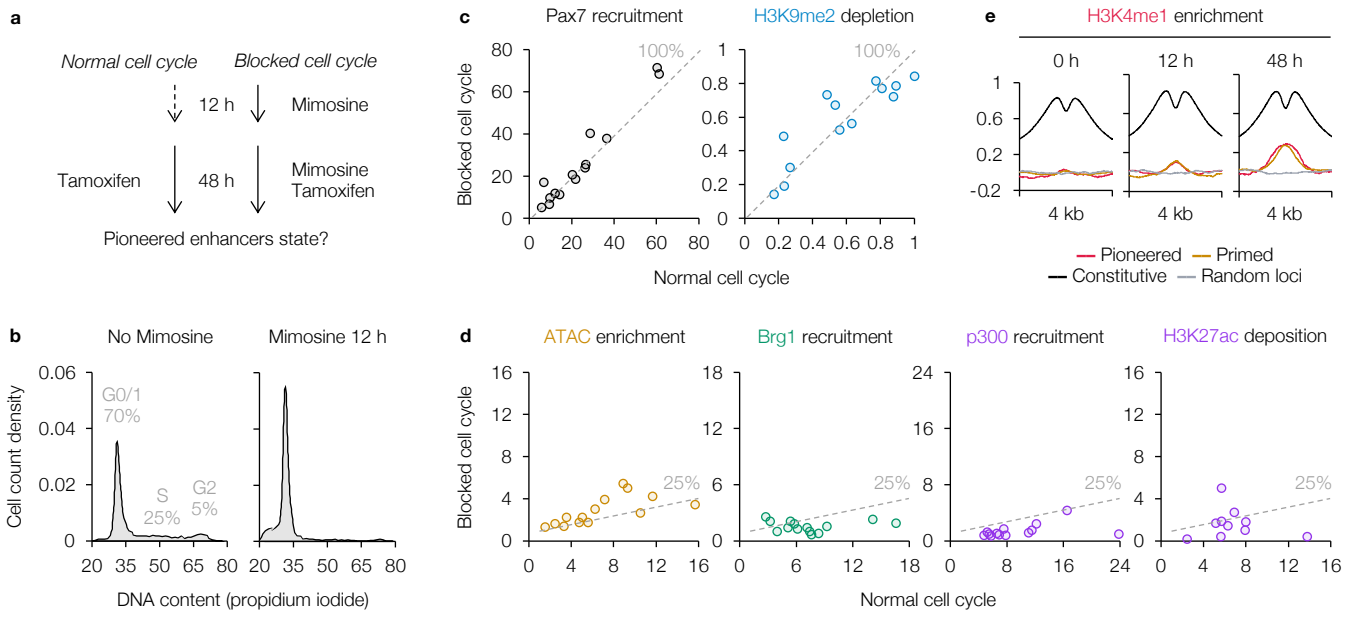

Fig. 3 | Activation of Pax7 pioneered enhancers requires cell division.

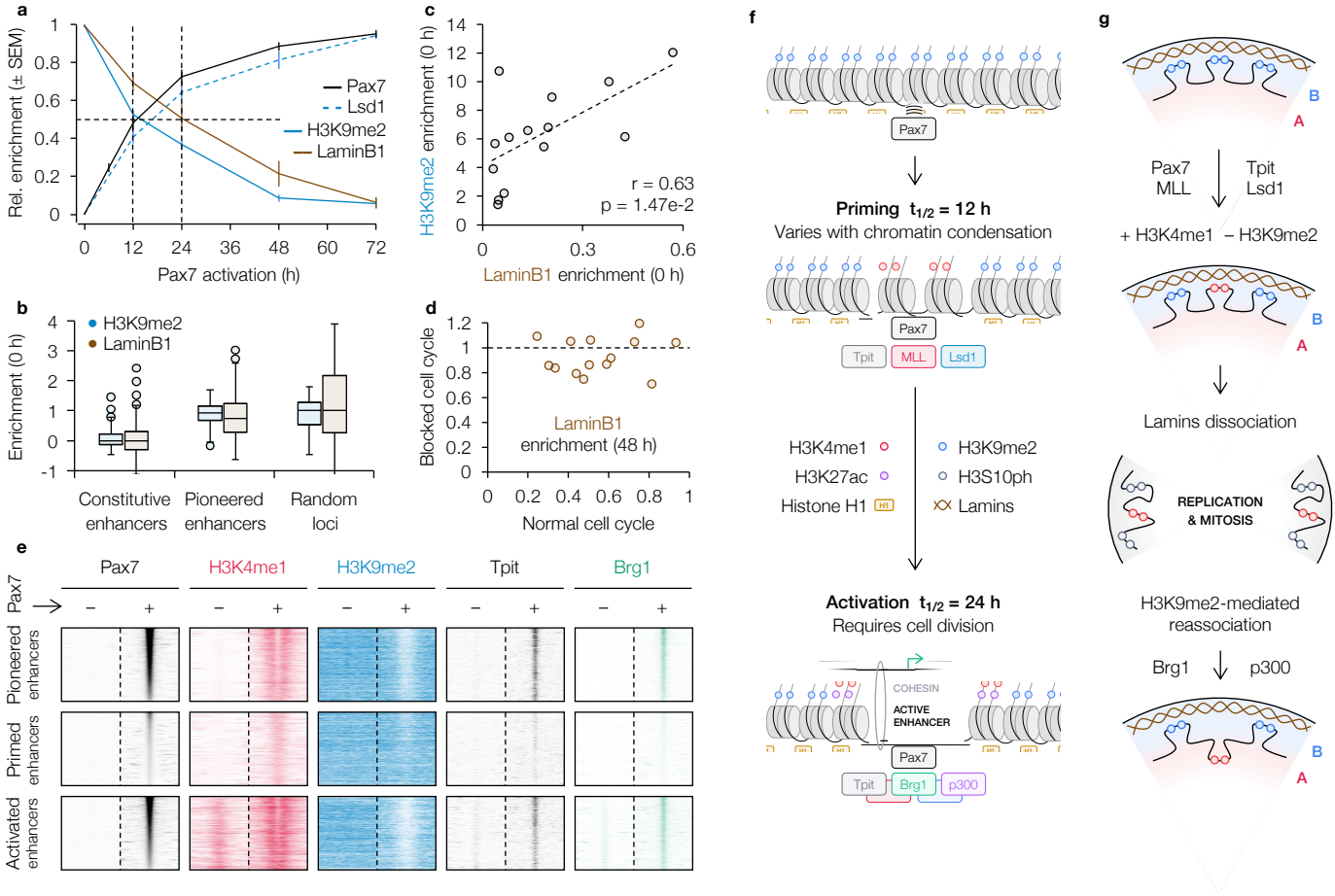

Fig. 4 | Pax7-dependent chromatin remodelling involves H3K9me2 demethylation and dissociation from lamins.