

Spatiotemporal regulation of multipotency during prostate development

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Summary statement

In this article we uncover the how multipotency of basal stem cells is regulated during prostate development using whole tissue imaging.

Abstract

The prostate is formed by a branched glandular epithelium composed of basal cells (BCs) and luminal cells (LCs). Multipotent and unipotent stem cells (SCs) mediate the initial steps of prostate development whereas BCs and LCs are self-sustained in adult mice by unipotent lineage-restricted SCs. The spatiotemporal regulation of SC fate and the switch from multipotency to unipotency remain poorly characterised. Here, by combining lineage tracing, whole tissue imaging, clonal analysis and proliferation kinetics, we uncover the cellular dynamics that orchestrate prostate postnatal development. We found that at the early step of development, multipotent basal SCs are located throughout the epithelium, and are progressively restricted at the distal tip of the ducts, where together with their progeny set up the different branches and the final structure of prostate. In contrast, pubertal development is mediated by unipotent lineage-restricted SCs. Our results uncover the spatiotemporal regulation of the switch from multipotency to unipotency during prostate development.

Introduction

The prostate is a glandular organ of the male reproductive system in mammals. It resides around the urethra and underneath the bladder (Abate-Shen and Shen, 2000; Marker et al., 2003). Its main function is to produce approximately one third of the seminal fluid. By supplying essential nutrients, enzymes and ions, prostatic secretions ensure the survival of the spermatozoa until the reproduction.

The adult prostate epithelium in rodents and humans is comprised of three distinct lineages: basal cells (BCs), luminal cells (LCs), as well as rare neuroendocrine cells (Abate-Shen and Shen, 2000). BCs form a discontinuous layer of cells characterised by the expression of K5, K14 and p63 surrounding the LC layer (Kasper, 2008; Shen and Abate-Shen, 2010). LCs express keratin K8, K18, androgen receptor (AR) and secretory proteins like prostate specific antigen (PSA) (Abate-Shen and Shen, 2000; Vashchenko and Abrahamsson, 2005). Few cells expressing both basal and luminal markers simultaneously, called intermediate cells, are presented in the developing and adult prostate in mice and humans (Garraway et al., 2003; Hudson et al., 2001; Taylor et al., 2010).

The mouse prostate is organised bilaterally different lobes including the ventral prostate (VP), the dorsolateral prostate (DLP) and the anterior prostate (AP) (Abate-Shen and Shen, 2000; Marker et al., 2003). The epithelium forms glands that are organised in ductal-branched structures. The VP is found below the bladder on top of the urethra, while the DLP is extended on each side of the urethra and the AP is located along the seminal vesicles. The prostate derives from epithelial budding of the endodermal urogenital sinus during embryonic development (Cunha et al., 1987; Timms et al., 1995). The urogenital sinus is committed to form prostatic epithelium on embryonic day 16 (E16) and the first epithelial buds can be observed at E17.5 (Timms et al., 1994). These buds grow into the mesenchyme generating the different lobes. The main ducts of each lobe are visible during embryonic development but they start to branch extensively after birth (Marker et al., 2003). By 8-10 weeks postnatally the mouse prostate development is entirely complete (Sugimura et al., 1986).

Lineage tracing experiments have shown that the cellular turnover in adult prostate during homeostasis and regeneration is mediated by distinct populations of unipotent basal and luminal restricted SCs (Choi et al., 2012). In addition, rare bipotent basal and luminal SCs were also observed when lineage tracing was

conducted with K5+ BCs or Nkx3-1+ LCs in adult mice during cycles of prostate regeneration following androgen administration (Lu et al., 2013; Wang et al., 2013). Genetic lineage tracing experiments performed at the early state of postnatal development (postnatal day 1 - P1) using K5 and K14 as well as K8 and K18-CreER to target the basal and luminal lineage respectively have been used to decipher the cellular and lineage hierarchy that controls prostate postnatal development (Ousset et al., 2012). This study revealed that BCs were heterogeneous during prostate postnatal development and contained basal multipotent SCs that differentiate into BCs and LCs, as well as unipotent basal and luminal progenitors. Mathematical modeling suggested that the apparent cellular heterogeneity of basal progenitors could be explained by stochastic cell fate decision of a single multipotent progenitor (Ousset et al., 2012). The lack of information regarding the clone location along the ductal trees as well as the lineage tracing at a single time point at P1 precluded from drawing definitive conclusions regarding the spatiotemporal regulation of cell fate during prostate postnatal development.

In this study, we combined different lineage tracing strategies and developed whole mount (WM) imaging techniques to analyse the heterogeneity of basal and luminal SCs and monitor their fate across the entire prostate epithelium at different time points during prostate postnatal development. This approach allows us to assess whether SC fate, such as multipotency and unipotency, is regulated spatially and temporally and determine when and where basal progenitors become lineage restricted during prostate postnatal development. We found that the proliferation and the fate of basal SCs change rapidly in a spatially and temporally regulated manner ensuring the harmonious postnatal growth of the prostate.

Results

Morphometric analysis of prostate postnatal development

The extensive postnatal growth of the murine prostate is mediated by SC activity that converts the embryonic prostate rudiment into a functional mature branched ductal network (Toivanen and Shen, 2017). How this postnatal SC activity is regulated remains currently poorly understood. In mice the neonatal development starts from P1 till P20, followed by the weaning (P21-P28) and puberty (P28-P42), while after males reach adult size and sexual maturity (P42-P60). To gain better insight into the epithelial architecture and the tubular network organisation of the developing prostate, we established a micro-dissecting protocol allowing isolation, staining, mounting and imaging of intact prostate lobes at different time points during postnatal development (Fig. 1A-D). We focused our analysis on the VP since its postnatal development begins immediately after birth (Timms et al., 1994). At P1, the VP had already undergone 1 to 3 bifurcations/branches and budding of new branches was clearly visible (Fig. 1A). The main branching events occurred during the first 15 days postnatally, while after P15 the number of ducts did not further increase and the ducts grew only in size until reaching their final adult structure around 6-8 weeks postnatally (Fig. 1B-1D). To understand the dynamics of tissue growth, we quantified several parameters that define precisely the overall tissue expansion. In particular, we measured the total length of all the ducts composing the VP and the width of the epithelium at distinct regions at different times during postnatal prostate development (Fig. 1E,G). We observed a linear tissue elongation until the onset of puberty, which usually takes place after weaning from P21 to P28 (Fig. 1F). The rate of growth increased between P21 and P42, which corresponds to the end of puberty, and then it decelerated until the length of the ductal tree reached a plateau (Fig. 1F). We measured the width of the ducts at the proximal, the intermediate, and the distal part as well as at the branching points (Fig. 1G). In all cases, we found that the width did not expand much until P21 and then increased from P21 to P42 to reach a plateau (Fig. 1H). To capture the evolution of the branching complexity of the ductal tree, we scored the total number of branching points and tips per prostate. We found that the number of branching points was increasing during the first 2 weeks of development while after P15, the total number of branching points was not further enhanced (Fig. 1I). The total number of tips continued to increase until the beginning of pubertal development (Fig. 1J) showing

that new buds can emerge from the existing branching points (Fig 1K). Confocal analysis of the tubular structures revealed that until P21, the tubes were composed of a single layer of LCs expressing K8 covered by a continuous layer of BCs expressing K5/K14 (Fig. 1L,M). However, from P21 to P42, the growth was accompanied by a folding of the epithelium into the inner part of the tubes increasing the area of LCs per duct and giving the appearance of tubular cavitation (Fig. 1N,O). This feature was observed along the whole length of the ducts. The increase in the width of the ducts was accompanied by the stretching and elongation of BCs, leading to the formation of a discontinuous BC layer (Fig. 1N,O). By scoring the total number of BCs and LCs per imaging field, we observed that the ratio of LCs/BCs was about 1:1 until puberty and then increased to 6:1 at P33 and 10:1 at P42-P56 (Fig. 1P,Q). This morphological change coincides with the onset puberty, suggesting that the hormonal alterations associated with this period induce a new mode of tissue morphogenesis that maximises the number of LCs per duct and the secretory capacity of the organ.

Altogether, these morphometric data show that the global architecture of the future adult prostate is established during the first part of the postnatal development until the beginning of the puberty, whereas the greatest tissue expansion and growth occur during pubertal development until adulthood.

Spatiotemporal regulation of cell division during postnatal development

To unravel the proliferation kinetics and whether cell proliferation is coupled with tissue growth during prostate postnatal development, we administrated EdU and evaluated its incorporation after a 12- or 24-hours chase by WM immunostaining. At P1, proliferation was equally elevated both in BCs and LCs, with more than 60% of EdU positive cells 12 hours following EdU administration (Fig. 2A,C). At P5, cell proliferation continued to be intense, although BCs were proliferating less frequently than LCs (Fig. 2B,C). Stromal cells presented high levels of proliferation at these two time points as well (Fig. 2C). The difference in proliferation between BCs and LCs was observed throughout the remaining postnatal growth. Around P10, there was a higher proliferation rate at the tip region (Fig. 2Q) as compared to the ductal region both in BCs and LCs, demonstrating that tissue proliferation is not stochastic all along the epithelium and is spatially regulated (Fig. 2D-F,P). The proliferation rate of stromal cells was similar at the tips and the ducts suggesting that the differences

between epithelial cells were not the consequence of a higher proliferation of stromal cells at the tip region (Fig. 2P). From the onset of puberty (P21) till adulthood (P56), our analysis revealed that BC, LC as well as stromal cell proliferation decreased overtime along the different regions until adult age, when both BCs and LCs proliferated at lower rate irrespectively of tissue localisation (Fig. 2G-Q).

We then questioned whether the increase of LC/BC ratio observed during development could be a result of differential cell death between BCs and LCs. We found that the overall tissue apoptosis was very low for both the basal and the luminal compartment, with a higher cell death rate in LCs, showing that the increase of LC/BC ratio is not a result of preferential BC death (Fig. S1A-D).

Spatiotemporal regulation of BC multipotency during prostate development

To assess the fate and multipotency of BCs during postnatal prostate development at organ level we performed Doxycycline (DOX) inducible lineage tracing of BCs using K14rtTA/TeO-Cre/Rosa-YFP mice at different time points of postnatal development and YFP expression was monitored by WM confocal analysis (Fig. 3A,B,H,N,T). This genetic strategy provided us the advantage to achieve high rate of BC recombination (at least 80% of YFP+ BCs) and define BC fate behaviour in relation to their relative position along the ductal tree. Administration of DOX at P1 and WM analysis one day later showed that only BCs were YFP labelled demonstrating the specificity of labelling using this approach (Fig. S1E,F). When we labelled BCs from P1 to P5 and examined the samples after DOX treatment (P5) and after 2 weeks of chase (P19), we observed an extensive contribution of BCs to the luminal lineage all along the ductal tree indicating that multipotent BCs were presented in all ductal regions during this early step of postnatal development (Fig. 3B-G). Next, we administrated DOX between P10-P15 and performed our analysis at P15 or after 2 weeks of chase (P29) (Fig. 3H). While the initial labelling was uniform along the ductal tree, we only found signs of multipotent fate at the tips as pointed out by the presence of many YFP+ K8+ LCs (Fig. 3I-M). These results show the progressive spatial restriction of basal multipotency to the distal tip and the lineage restriction of BCs along the duct occurs as soon as P10. DOX administration from P16-P21 led to the labelling of rare LCs after 2 weeks of chase (P35) (Fig. 3N-S), while BC tracing from P21-P26 led only to BC labelling after 2 weeks chase (P40) suggesting that BCs did not differentiate anymore to the luminal lineage (Figure 3T-

Y). The progressive switch from multipotency to unipotency was also observed in the other prostate lobes (Data not shown). These data demonstrate that BCs undergo a switch from multipotency to unipotency relatively early during prostate postnatal development, at the onset of puberty, and this lineage restriction occurs in a spatially graded manner with the tips being the multipotent niche during the mid-stage of postnatal development.

Widespread distribution of multipotent and luminal committed basal SCs at the early stage of postnatal development

Our previous short term clonal analysis of prostate BCs induced at P1 by imaging tissue sections suggested that either multiple types of multipotent and unipotent SCs co-exist at birth or that a single population of multipotent SCs divides asymmetrically giving rise to a multipotent SC and a unipotent luminal SC (Ousset et al., 2012). However, the absence of 3D information of the whole prostate and the single time point at which we performed the clonal analysis (P1), prevented us from discriminating between these two possibilities. To investigate more precisely the heterogeneity of cell fate and the clonal dynamics of BCs during the early stage of postnatal development, we performed lineage tracing of BCs at clonal density at P1 and assessed their fate all along the ductal tree after 3 weeks and 6 weeks of chase using WM confocal microscopy. We used K5CreER^{T2} mice, since K5 is a cytokeratin expressed specifically in all BCs at the early stage of prostate development and took advantage of the four colour confetti construct to evaluate the clonality of our labelling strategy (Fig. 4A). We administered a low dose of tamoxifen (TAM) to P1 mice and scored the recombination frequency of the cells at different time points post induction (Fig. 4B). We observed isolated BCs expressing one of the four confetti colours 3.5 days after labelling irrespectively of their relative position within the ductal tree (Fig. 4C,D). The analysis of fluorescent patches in WM 1, 3 and 6 weeks after induction demonstrated the presence of clusters of fluorescently labelled cells containing BCs and/or LCs along the ducts and the tip regions (Fig. 4E-L and Fig. S2A,D,E).

Soon after initial labelling (P7), fluorescent marked clones formed small cluster of labelled cells that are cohesive (Fig. S2A). At later time points (P21, P42), these fluorescent labelled cells were often not cohesive and were separated by non-labelled cells (Fig. 4E,I and Fig. S2D,E). To validate the clonality of our tracing

experiments at P21 and P42, we scored the recombination frequency of each fluorescent protein (YFP, RFP, mCFP, nGFP) and the number of colours observed per ductal subtree. A subtree is defined as the different branches that share a common ancestor at the level of the second branch of a ductal tree, which exists prior to TAM administration (Fig. 4M-O). We found that 3 and 6 weeks after TAM administration, 60% of all subtrees were not labelled at all, the vast majority of labelled subtrees (>80%) were labelled only with one confetti colour and the minority of the ductal trees were labelled by more than one colours (Fig. 4O and Fig. S1F,G), indicating that the fluorescent patches of the same colour within a ductal tree are of clonal origin. Interestingly, clones of common origin were often separated by non labelled cells (clone fragmentation) and appeared as non-continuous areas of labelled cells participating in the morphogenesis of several branches along the ductal tree, sometimes composed of different cell types depending on the branches (Fig. 4E,I).

To analyse the fate of BCs, we recorded the cellular composition of all clones and their location within the ductal tree 1, 3 and 6 weeks after induction. One week after labelling, 40% of clones were bipotent, 22% contained only LCs and 38% were composed only of BCs (Fig. S2B). At 3 and 6 weeks after induction, 60% of the clones were bipotent containing BCs and LCs, 35% were composed exclusively of LCs and less than 5% contained only BCs (Fig. 4P). The relative proportion of bipotent and unipotent clones did not change between 3 and 6 weeks post induction (Fig. 4P).

Next, we quantified the number of BCs and LCs in bipotent and unipotent clones. The number of BCs per clone was relatively homogeneous with no particularly big clones and the mean basal clone size was 3 cells at 1 week tracing, 6 cells and 7 cells at 3 and 6 weeks respectively (Fig. 4Q and Fig. S2C,H,I). These data suggest that the basal compartment expanded by 3 folds from P1 to P7 and by 2 folds from P7 to P21, while it did not increase further from 3 to 6 weeks, consistent with the decrease in cell density of BCs found at the onset of puberty (Fig. 4Q and Fig. 1O). The number of LCs per clone presented a much greater heterogeneity ranging from small clones containing few LCs to clones composed of more than 150 LCs at 3 weeks until 400-600 LCs at 6 weeks (Fig. 4Q and Fig. 2H,I). The mean number of LCs per clone was 5 cells at 1 week, 36 cells at 3 weeks and 141 cells at 6 weeks, showing the considerable and continuous expansion of the LC lineage

during the whole process of prostate postnatal development until adulthood (Fig. 4Q and Fig. 2C).

To assess whether the clonal dynamic is influenced by its position along the ductal tree, we plotted the number of cells per fragment according to its position in relation to the end of tip (0 μ m). We found that fragment size did not have a strong correlation with its position along the duct (Fig. 4R,S), further suggesting that the SC activity drives the overall tissue expansion during early development.

Altogether, these data show that BCs consist of a heterogeneous population already at P1, with the majority of the BCs being multipotent and the rest committed to the LC lineage. Only a very small proportion of BCs remains purely basal during the course of prostate development.

Multipotency is restricted to the tip region during mid postnatal prostate development

To verify whether the fate and the dynamics of BCs change during the course of postnatal prostate development, we performed clonal lineage tracing of BCs at later time points, at P12 (Fig. 5) and P21 (Fig. 6), and assessed the fate outcome of BCs 3 weeks and 6 weeks following TAM administration to K5CreER/Rosa-Confetti mice. Similarly to what we observed at the clonal tracing performed at P1, only isolated labelled BCs with no preference for the duct or the tip were observed at 3.5 days and 1 week after tracing at P12 (Fig. 5B-D). The analysis of WMs 3 and 6 weeks after induction showed the presence of clusters of fluorescently labelled cells containing BCs and/or LCs mainly at the tip regions (Fig. 5E-G,I-K and Fig. S3A,B). In addition, clusters containing only labelled BCs were observed (Fig. 5H,L). As in our P1 clonal tracing, the vast majority of tips were not fluorescently marked while most of the labelled tips contained only one colour, demonstrating the clonality of these lineage experiments (Fig. 5M, 5N and Fig. S2C,D).

The clone composition (% and # of BCs and LCs per clone) was determined by confocal microscopy of WMs 3 weeks and 6 weeks after induction. We scored the clone composition according to their relative position along the ductal tree. For these measurements, we segmented the tissue into 2 different regions including the distal part of the duct (Distal tip - up to 500 μ m), and the remaining duct (Duct - greater than 600 μ m from the tip). Most of the clones with bipotent basal and luminal fate or only luminal fate were found around the distal tip 3 and 6 weeks after BC labelling at

P12, suggesting that multipotent BCs are localised exclusively close to the tip region at this stage of development while BCs along the duct are already lineage restricted (Fig. 5O). Moreover, the percentage of bipotent and luminal clones located along the ducts increased 6 weeks after induction (Fig. 5O), suggesting that the net growth of the duct arises also from the production of cells at the tip region that progressively transfer the newly generated LCs in a more proximal region of the duct.

The average number of BCs per clone was very small (1-6 BCs) in comparison to the average number of LCs per clone that contained approximately 80 LCs and 106 LCs 3 and 6 weeks after P12-induction respectively (Fig. 5P and Fig. S3E,F). Interestingly, some clones contained more than 200 LCs. The clones with the higher number of BCs were unipotent with no LCs (Fig. S3E,F), demonstrating that unipotent and multipotent BCs could divide at the same rate, with unipotent BCs dividing symmetrically and multipotent BCs dividing mostly asymmetrically giving rise to a BC and a LC, leading to the expansion of the pool of LC progenitors. The number of cells per clone increased with the proximity to the tip region (Fig. 5Q,R), supporting the notion that multipotent SC activity around P12 is located around the tip region and responsible for the net local expansion of the prostate epithelium.

Basal stem cells become unipotent at the onset of puberty

Clonal tracing in K5CreER/Rosa-Confetti mice at P21 and analysis 1 week after induction showed the initial labelling of BCs along the duct at very low density (Fig. 6A-D). Analysis performed 3 weeks (P42) and 6 weeks (P63) after induction demonstrated that most BCs are unipotent regardless their location along the duct or at the tip region (Fig. 6E-J,M,N and Fig. S2G,H). Moreover, less than 10% of all clones presented some LCs while the number of LCs per clone was rather limited to an average of 5 and 8 cells 3 and 6 weeks after induction (Fig. 6K,L). These findings show that the important BC contribution to the luminal lineage ends after P21 and suggest that LC expansion, which accompanies puberty, is mediated essentially by unipotent luminal SCs that were specified earlier during development.

Important contribution of unipotent luminal SCs to LC expansion during postnatal development

To directly determine the importance of unipotent luminal SCs in ensuring luminal lineage expansion during prostate postnatal development, we performed clonal analysis of luminal-targeted cells using K8rtTA/TetO-Cre/Rosa-Confetti mice at different time points during postnatal development (Fig. 7A). Administration of DOX to mice at P1 led to luminal clones of various sizes ranging from 10-50 LCs (26 LCs in average) 3 weeks after induction and from 20-120 LCs (68 LCs in average) 6 weeks after induction (Fig. 7B-E and Fig. S3A,B). Interestingly, the size of the luminal clones was not bigger close to the tip region and clones were evenly distributed along the ductal tree (Fig. 7F-G), suggesting that once specified, the different luminal progenitors contribute importantly to the net expansion of the LC lineage during postnatal growth.

DOX administration at later time points (P12 and P21) similarly led to the formation of luminal clones of various size located along the ductal tree 3 weeks (P33 and P42) and 6 weeks (P54 and P63) post induction (Fig. 7H-S and Fig. S2C-F). Interestingly, the average size of the clones at 6 weeks decreased with the time of DOX administration (dropping from 68 to 41 – Fig. 7E,K,Q), consistent with the morphometric analysis showing that most of the prostate postnatal expansion occurs before P42.

Discussion

In this study, we uncover the spatiotemporal regulation of the proliferation dynamics and multipotency during prostate postnatal development.

Our whole tissue imaging provided insightful information regarding prostate growth. We showed that early postnatal development is accompanied by a linear increase both in tissue size (length and width) and complexity (branching morphogenesis). The onset of puberty coincides with faster growth rate and expansion of the luminal compartment, suggesting that the hormonal changes during this period is associated with the induction of a new mode of tissue morphogenesis that maximises the secretory capacity of the organ as it reaches sexual maturity.

Using different lineage tracing approaches induced at different time points during postnatal development, we demonstrate that the majority of basal SCs are multipotent or committed to the luminal lineage during the first days of prostate postnatal development. At that stage of postnatal development multipotent basal SCs are present all along the ductal trees, giving rise to luminal progenitors, which present extensive self-renewal capacities, expansion and differentiation potential. At that time, BCs comprise also committed luminal SCs or progenitors that give rise only to LCs, without any trace of their previous BC fate. As prostate development proceeds, the main ductal regions lose their multipotent fate and multipotent BCs become located exclusively at the tip regions, promoting the prostate remodelling and branching that precedes prostate growth during puberty. Once the final pattern of the future adult prostate is established, with all the definitive branches and tips, the final prostate expansion, which is associated with puberty, is mediated mainly by unipotent basal and unipotent luminal SCs and progenitors. In prostate, this switch from multipotency to unipotency occurs in the childhood before puberty. In contrast, in MG development which presents more advanced branching at birth as compared to the prostate, the switch from multipotency to unipotency of BCs occurs during embryonic development between E15.5 and E18.5 and all postnatal development is mediated by unipotent SC in the MG (Wuidart et al., 2018; Lilja et al., 2018). Moreover, LCs of the MG comprise at least two distinct lineages expressing or not estrogen and progesterone receptors. Sweat glands are also initially developed by different classes of multipotent and lineage restricted cells and become unipotent around 4 weeks of development (Lu et al., 2012). Interestingly, these different studies show that whereas various glandular epithelia present many similarities in the lineage differentiation potential of basal SCs switching from multipotent to unipotent SCs during the course of development, the temporal clock regulating this process is not identical among the different epithelia. It would be interesting to assess in the future the mechanisms that regulate the temporal clock, and whether the mechanisms that restrict or activate multipotency are common across different glandular epithelia.

Multipotency is also an important process during prostate tumorigenesis. When the most frequently mutated tumour suppressor gene, Pten, is deleted from BCs, they reactivate a multipotent program and begin to generate LCs before progressing into invasive tumours (Choi et al., 2012; Wang et al., 2013). Similar

reactivation of multipotency has also been observed during MG tumorigenesis following the expression of oncogenic *Pik3ca* in adult BCs or LCs (Van Keymeulen et al., 2015). The gene signature that accompanies the reactivation of multipotency in these cells following oncogene expression resembles the gene signature that marks the embryonic multipotent SCs (Wuidart et al., 2018; Lilja et al., 2018), supporting the notion that reactivation of an embryonic program and multipotency is a hallmark of tumorigenesis in glandular epithelia.

More studies are needed to better understand the reasons why multipotency is actively suppressed in adult animals in physiological conditions, and why and how this multilineage fate potential is unleashed and reactivated during tissue repair, transplantation and oncogenic activation. It would be interesting to understand the nature of the niche surrounding the tip region that regulates multipotency during the mid-stage of prostate development. Do tip cells secrete specific signals that promote multipotency of BCs? What are the roles of the stromal and immune cues in the regulation of multipotency?

In conclusion, we identify in this study the spatiotemporal regulation of prostate postnatal morphogenesis, the spatial organisation of multipotent SCs and the temporal switch from multipotency to unipotency. These results will be instrumental to unravel the molecular mechanisms that regulate multipotency during prostate development, regenerative conditions and tumorigenesis.

Materials and Methods

Mice

K14rtTA (Nguyen et al., 2006) mice were provided by Elaine Fuchs, TetO-Cre (Perl et al., 2002) were provided by Andreas Nagy and Rosa26-Confetti (Snippert et al., 2010) mice were provided by Hans Clevers. Rosa26-YFP (Srinivas et al., 2001). CD1 mice were obtained from the Jackson Laboratory. The generation of K5CreER^{T2} mice (Van Keymeulen et al., 2011) and K8rtTA mice (Watson et al., 2015) was previously described. The experimental mice used in this study were of male gender and mixed background. All animals were housed under standard laboratory conditions in a certified animal facility receiving food and water *ad libitum*. All experiments were conducted in compliance with the European guidelines and ethical

protocols (under protocol numbers 546N and 673N) were approved by the local ethical committee for animal welfare (CEBEA). No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Induction of YFP expression

For tracing at saturation, K14rtTA(h);TetO-Cre(h);R26-YFP(h) mice were induced with a single dose of doxycycline (DOX) (Sigma; diluted in PBS) administrated by intraperitoneal injection at P1 (20µl of 10 mg/ml), P10, P16 or P21 (100µl of 10 mg/ml) and maintained on treatment for 5 days by oral administration of DOX diluted in drinking water (2 mg/ml, AG Scientific) provided to them or to the mother. Mice were analyzed at the end of DOX treatment (no chase) or 2 weeks later (chase 2w). To validate leakiness of the system and not K14 specific labeling, P1 mice received a single dose of DOX by intraperitoneal injection (20µl of 10 mg/ml) and sacrificed one day later.

Induction of Confetti expression

For clonal lineage tracing of basal cells, K5CreER^{T2}(h);R26-Confetti(h) mice were induced with a single dose of Tamoxifen (TAM) (Sigma; diluted in sunflower seed oil, Sigma) administrated by intraperitoneal injection at P1 (20µl of 2.5 mg/ml), at P12 (100µl of 2 mg/ml) or at P21 (100µl of 10 mg/ml) and sacrificed 3.5 days, 1 week, 3 weeks or 6 weeks later. For clonal lineage tracing of luminal cells, K8rtTA(h);TetO-Cre(h);R26-Confetti(h) mice were induced with a single dose of DOX (diluted in PBS) administrated by intraperitoneal injection at P1 (20µl of 1 mg/ml), at P12 (100µl of 0.2 mg/ml) or at P21 (100µl of 0.8 mg/ml) and sacrificed 3 weeks or 6 weeks later.

Cell proliferation assay

To trace proliferating cells, mice at different age were injected intraperitoneally with 12.5 mg/kg 5-ethynyl-2'-deoxyuridine (EdU Molecular Probes; diluted PBS) every 12 hours. Animals were sacrificed 12 or 24 after the first injection.

Whole mount prostate processing and immunofluorescent staining

Samples processed at P1, P2, P3, P3.5 and P5 were obtained by dissection of the entire urogenital system followed by removal of the bladder, the testicles and the fat tissue. For samples processed at later time points, prostate tissue was microdissected under a stereoscope to separate the different lobes. The ventral lobes were enzymatically digested in HBSS (Gibco) supplemented with 10 mg/ml collagenase (Sigma) for 2 to 8 minutes at RT depending on tissue size and animal age. Samples were rinsed twice with PBS for 5 minutes and fixed at 4% paraformaldehyde (PFA) for 2 hours at RT. After 2 times of washing with PBS for 5 minutes, tissues were incubated in blocking buffer (1% bovine serum albumin (BSA), 5% horse serum (HS) 0.8% TritonX-100 in PBS) for 3 hours at RT while shaking. Primary antibodies were diluted in blocking buffer and incubated overnight at RT while shaking. Lobes were washed 3 times with PBS 0.2% Tween20, 10 minutes each, before being incubated for 4-5 hours at RT on a rocking plate with the proper secondary antibody diluted in blocking buffer. Anti-GFP (chicken, 1:1,000, ab13970, Abcam), anti-K14 (rabbit, 1:2,000, PRB-155P-0100, Covance), anti-K5 (chicken, 1:2,000, 905901, Covance) and anti-K8 TROMA-1 (rat, 1:800, Developmental Studies Hybridoma Bank) were used as primary antibodies. Anti-chicken, anti-rabbit and anti-rat conjugated to AlexaFluor488 (1:400, Molecular Probes), anti-rabbit conjugated to Rhodamine Red-X or to Cy5 (1:400, Jackson ImmunoResearch) and anti-chicken and anti-rat conjugated to Cy5 (1:400, Jackson ImmunoResearch) were used as secondary antibodies. To visualize proliferating cells that incorporated EdU, the Click-iT® EdU AlexaFluor594 Imaging Kit (Molecular Probes) was used and the Click-iT reaction was performed according to manufacturer's instructions but with extended permabilization to 0.8% TritonX-100 for 30' followed by 1.2% TritonX-100 for 10'. Subsequently, incubation to the EdU cocktail was extended to 40'. Nuclei were counterstained using Hoechst 33342 dye (Sigma) (diluted 1:10,000-Confetti mice or 1:1,000-YFP or EdU mice in PBS 0.2% Tween20) for 30 minutes (Confetti or YFP mice) or 2 hours (EdU mice) at RT while shaking. Tissues were washed twice in PBS 0.2% Tween20 for 10 minutes and mounted on a 1.5 mm coverslip (VWR) in glycerol mounting medium (DAKO) supplemented with 2.5% Dabco (Sigma).

Histology and immunostaining on sections

Prostate tissue of mice at P21, P42 and P56 was micro-dissected under a stereoscope to separate the different lobes. Dissected prostates were pre-fixed in 4% PFA for 2 hours at RT. After three times of washing with PBS for 5 minutes, samples were incubated overnight in 30% sucrose in PBS at 4°C. Tissues were embedded in OCT compound (Tissue Tek) and kept at -80°C. Sections of 5 µm were cut using a HM560 Microm cryostat (Mikron Instrument). Sections were incubated in blocking buffer (1% BSA, 5% HS, 0,2% TritonX-100 in PBS) for 1 hours at RT. Primary antibodies were incubated overnight at 4°C. Tissues were rinsed three times in PBS, 5 minutes each, and incubated with secondary antibodies diluted in blocking buffer for 1 hour at RT. Anti-K14 (chicken, 1:1,000, PRB-155P-0100, Covance), anti-K8 TROMA-1 (rat, 1:1,000, Developmental Studies Hybridoma Bank) and anti-cleaved caspase3 (Asp175) (rabbit 1:400, 9664S, Cell signaling) were used as primary antibodies. Anti-rat conjugated to AlexaFluor488 (1:400, Molecular Probes), anti-chicken conjugated to Rhodamine Red-X (1:400, Jackson ImmunoResearch) and anti-rabbit conjugated to Cy5 (1:400, Jackson ImmunoResearch). Nuclei were stained with Hoechst 33342 dye (Sigma) (diluted 1:1,000 with the secondary antibodies) and slides were mounted in glycergel mounting medium (DAKO) supplemented with 2,5% Dabco (Sigma).

Image acquisition and analysis

Confocal images were acquired at RT using a Zeiss LSM780 confocal microscope fitted on a Axiovert M200 inverted microscope equipped with a LD C Aplanachromat (40X, NA=1.1) water immersion objective (Carl Zeiss Inc.). Optical sections 512 x 512 pixels were collected sequentially for each fluorochrome. Images of maps reconstructing the whole mounts were obtained at RT using a LD LCI Plan Aplanachromat (25X, NA=0.8) water & glycerol immersion objective (Carl Zeiss Inc.) or a Plan Aplanachromat (20X, NA=0.8) without immersion objective (Carl Zeiss Inc.). Optical sections 512 x 512 or 256 x 256 pixels (depending on map size) were collected sequentially for each fluorochrome. The generated data were processed and displayed using ZEN software. Maps were cropped if needed to isolate the prostate epithelium from mesenchyme and surrounding tissue. Quantifications were performed manually using the scoring tool of ZEN software.

Clonal Analysis

Clone size and composition was checked and scored manually. To assess clonality in K5CreER^{T2}(h);R26-Confetti(h) mice induced at P1 after 3 and 6 weeks of tracing, the number of confetti colors per subtree and the color repartition were scored. Using the frequency of observing 0 colors per subtree, the probability of observing 1, 2, 3, and 4 colors per subtree was estimated with the poissonian distribution. To assess clonality in K5CreER^{T2}(h);R26-Confetti(h) mice induced at P12 after 3 and 6 weeks of tracing, the number of confetti colors per tip and the color repartition were scored. Using the frequency of observing 0 colors per tip, the probability of observing 1, 2, 3 and 4 colors per tip was estimated with the poissonian distribution. To assess clonality in K5CreER^{T2}(h);R26-Confetti(h) mice induced at P21, we macroscopically examined on maps that no marked cells were found in a distance of 500µm far from clones of marked cells. To assess clonality in K8rtTA(h);TetO-Cre(h);R26-Confetti(h) mice induced at P1, P12 or P21 after 3 and 6 weeks of tracing, only clones that were isolated at least 500µm from other labeled cells were scored.

Statistics

Statistical and graphical data analyses were performed using Prism 6 (Graphpad) software. All data in histograms represent mean ± SEM or ±SD. Data were tested for normality using D'Agostino and Pearson omnibus normality test. Statistical significance was calculated by Mann-Whitney, considering $p < 0.05$ as statistically significant. R^2 was calculated by computing Pearson correlation coefficient. All tests were two-sided.

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Author Contributions

E.T., M.O. and C.B. designed the experiments and performed data analysis. E.T. conducted most of the experiments. M.O. conducted some of them and made initial observations. A.D. provided technical support. E.T. prepared the figures. C.B. and E.T wrote the manuscript.

Competing interests

The authors declare no competing interests.

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Figures

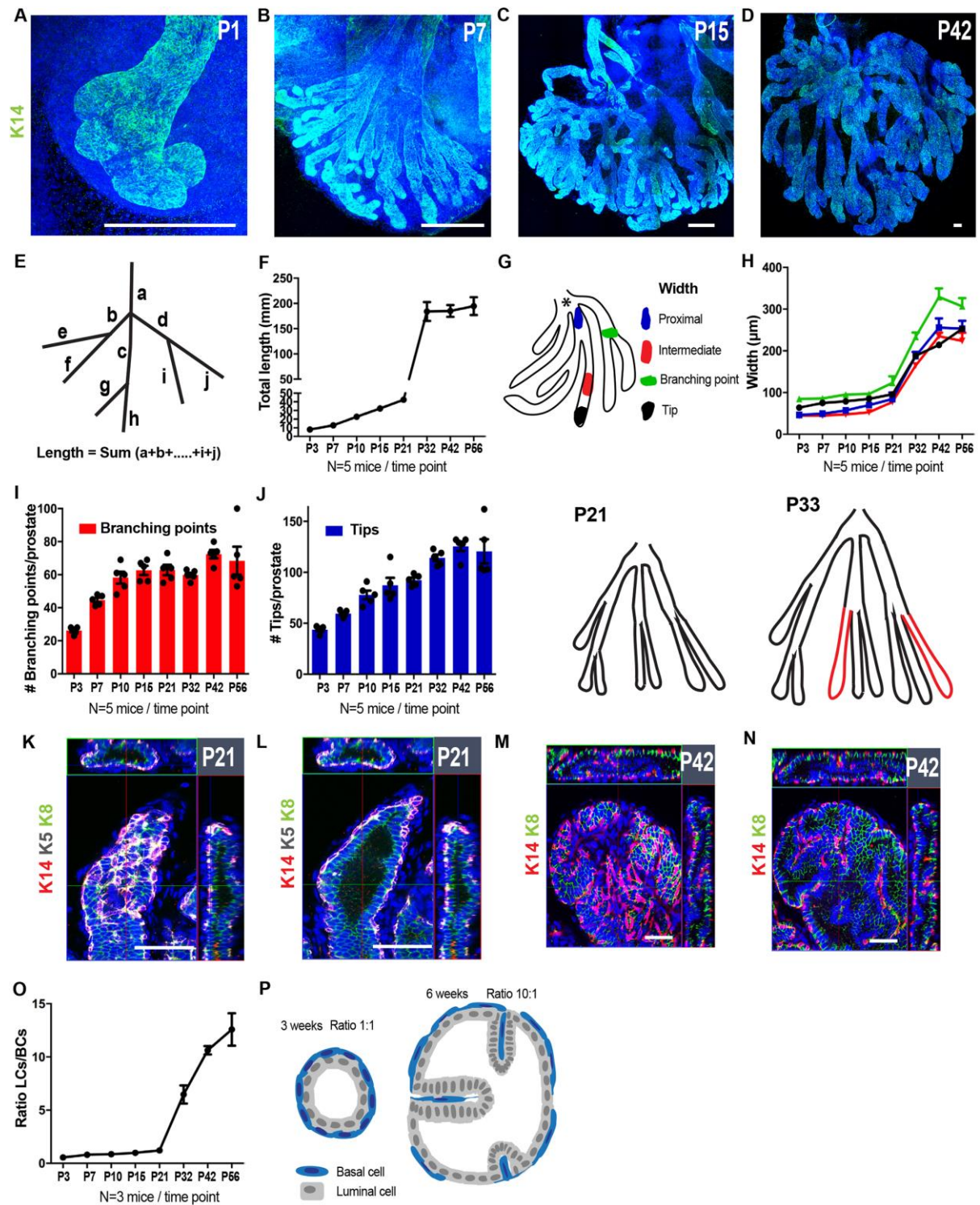


Fig. 1. Three-dimensional analysis of prostate postnatal development.

(A-D) Three dimensional confocal microscopy analysis of WM of the VP at different time points during postnatal development. Tissue is stained with K14 (green) and Hoechst (blue). (E) Schematic illustration of the ductal tree showing the method used to measure the length of the prostate epithelium in F. (F) Measurement of the total length of the ductal tree at different times. (G) Schematic illustration of the different ductal regions, where width was measured in H. (H) Measurement of the width of the different regions of the ductal tree at different times. Five different measurements were taken per region, per sample. (I) Total number of branching points per VP at different time points. (J) Total number of tips per ventral prostate at different times. (K) Schematic illustration of the progression in the number of branching points and tips at P21 and P33. (L-O) Confocal images at P21 (L,M) and at P42 (N,O) at the upper layer (L & N) and the middle layer of the ductal epithelium (M & O) stained with K14 (red), K5 (grey)-(in L & M), K8 (green) and Hoechst (blue), showing the stretching of the basal compartment and progressive cavitation of the luminal compartment. (P) Ratio of LCs to BCs in the prostate epithelium at different time points. In total 13,761, 23,447, 20,706, 30,756, 59,386, 48,658, 54,101 and 63,199 cells were counted for each time point. (Q) Scheme illustrating a cross section of a prostate duct at P21 (left) and P42 (right). Data show mean \pm SD (F,I,J) and mean \pm SEM (H,P). Scale bar, 200 μ m (A-D) and 50 μ m (L-O).

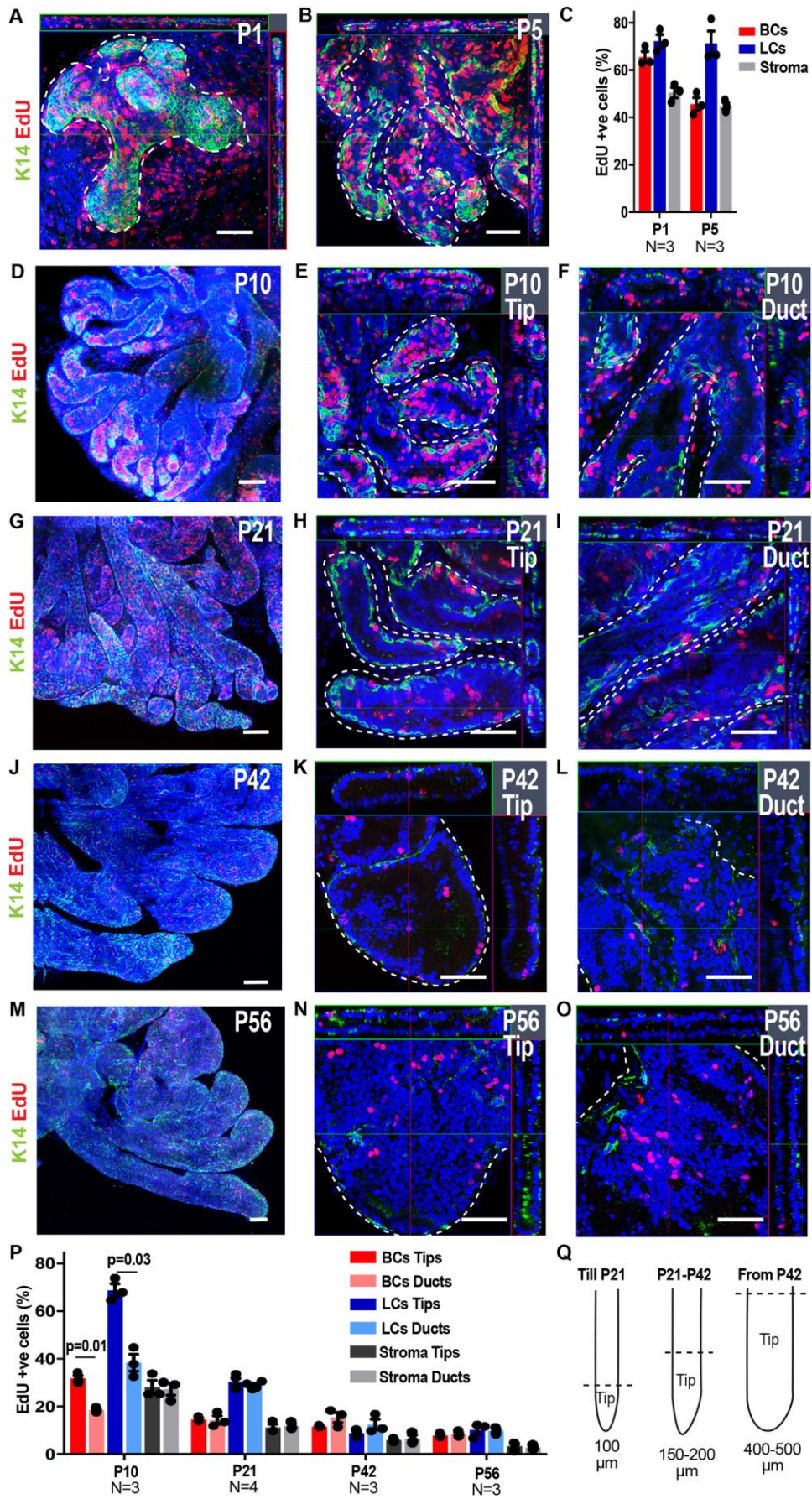


Fig. 2. Spatiotemporal regulation of cell proliferation during prostate postnatal development.

(A,B) Confocal images of EdU incorporation in the VP at P1 (A) and P5 (B). (C) Quantification of proportion of EdU positive cells at P1 and P5 12 hours following EdU administration. Mean \pm SEM are shown from 6,772 and 8,928 cells counted from 3 mice for P1 and P5 respectively. (D-O) Confocal images of the proliferating cells at low magnification (D,G,J,M), at the tip (E,H,K,N) and at the duct (F,I,L,O) of the VP at different points during development. (P) Quantification of EdU positive cells in tips and ducts 24 hours following EdU administration at different time points during postnatal development. Mean \pm SEM are shown from 31,916, 30,388, 27,759 and 21,029 cells counted at the different time points indicated. (Q) Schematic illustration showing the distance of the distal tip of the ducts that is defined as the tip region for data analysis at different time points during development. In all panels from B-P K14 is visualized in green, EdU in red and nuclei were counterstained with Hoechst in blue. Dashed lines outline the ducts. Scale bar, 50 μ m (A,B,E,F,H,I,K,L,N,O) and 100 μ m (D,G,J,M). P-values were calculated by Mann-Whitney test.

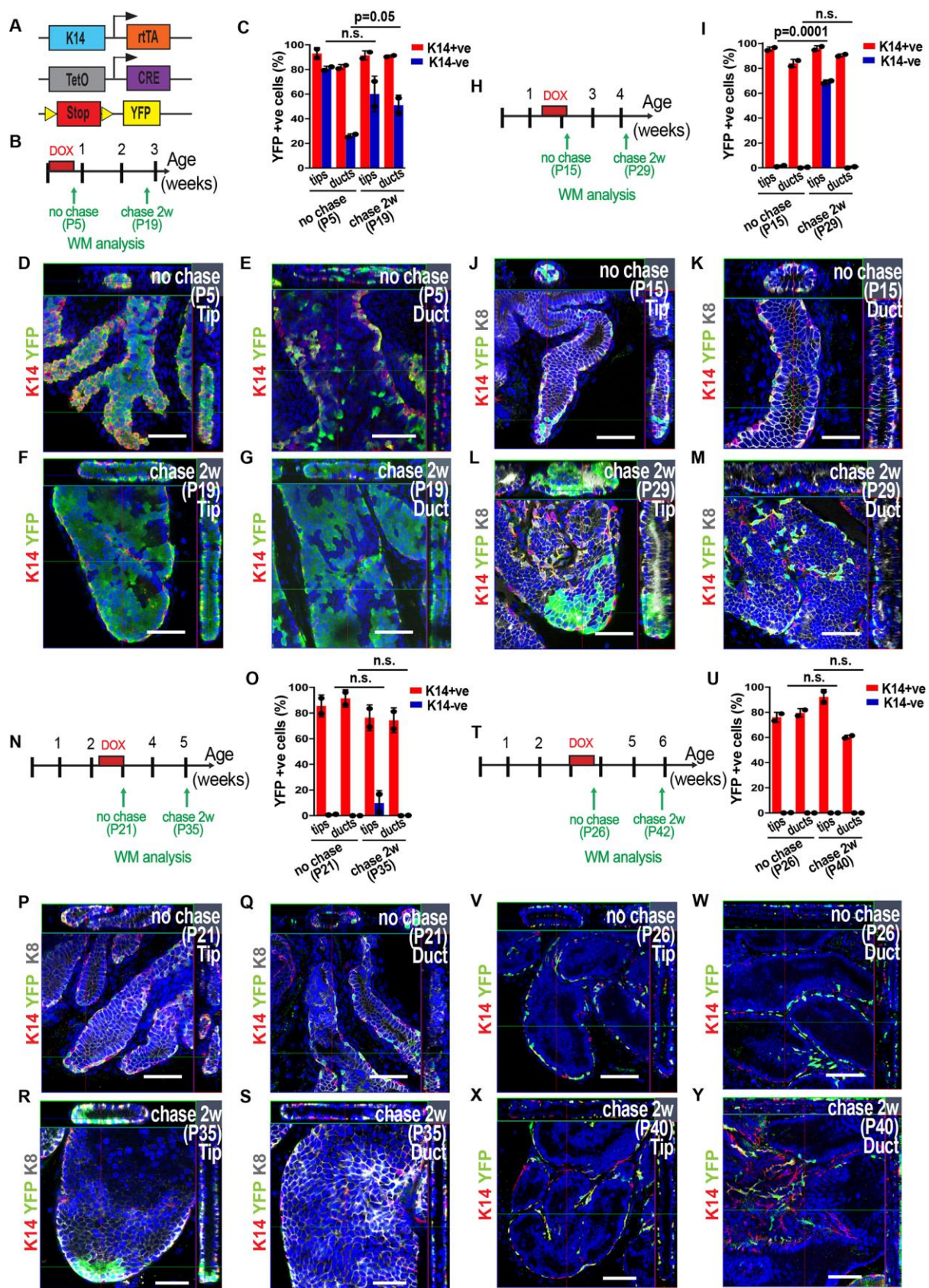


Fig. 3. Basal lineage tracing at saturation shows the spatiotemporal regulation of multipotency during prostate development.

(A) Genetic strategy used to induce YFP expression in most K14 expressing BCs at different time points during prostate development. (B) Protocol used to analyse saturation lineage tracing at early stage of postnatal development (P1-P5). (C) Quantification of YFP+ cells in BCs (K14+) and LCs (K14-) at the end of DOX administration (P5) and after 2 weeks of chase (P19) at the tip or the duct. In total 19,322 and 23,298 cells from 2 mice were counted at the indicated time point. (D-G) Confocal images of the VP at the end of Dox administration (P5) (D,E) and after 2 weeks of chase (P19) (F,G). (H) Protocol used to analyse saturation lineage tracing during mid postnatal development (P10-P15). (I) Quantification of YFP+ cells in BCs (K14+) and LCs (K14-) at the end of Dox administration (P15) and after 2 weeks of chase (P29) at the tip or the duct. In total 23,362 and 24,712 cells from 2 mice were counted at the indicated time point. (J-M) Confocal images of the VP at the end of treatment (P15) (J,K) and after 2 weeks of chase (P29) (L,M). (N) Protocol used to analyse saturation lineage tracing performed just before puberty (P16-P21). (O) Quantification of YFP+ cells in BCs (K14+) and LCs (K14-) at the end of Dox administration (P21) and after 2 weeks of chase (P35) at the tip or the duct. In total 44,114 and 36,544 cells from mice were counted at the indicated time point. (P-S) Confocal images of the VP at the end of Dox administration (P21) (P,Q) and after 2 weeks of chase (P35) (R,S). (T) Protocol used to analyse saturation lineage tracing performed at the onset of puberty (P21-P26). (U) Quantification of YFP+ cells in BCs (K14+) and LCs (K14-) at the end of Dox administration (P26) and after 2 weeks of chase (P40) at the tip or the duct. In total 51,064 and 44,781 cells from 2 mice were counted at the indicated time point. (V-Y) Confocal images of the VP at the end of Dox administration (P26) (V, W) and after 2 weeks of chase (P40) (X, Y). In all panels K14 is visualised in red, YFP in green, K8 in grey (in J-M, P-S) and nuclei were counterstained with Hoechst in blue. Data show mean±SEM. Scale bar, 50µm. P-values were calculated by Mann-Whitney test.

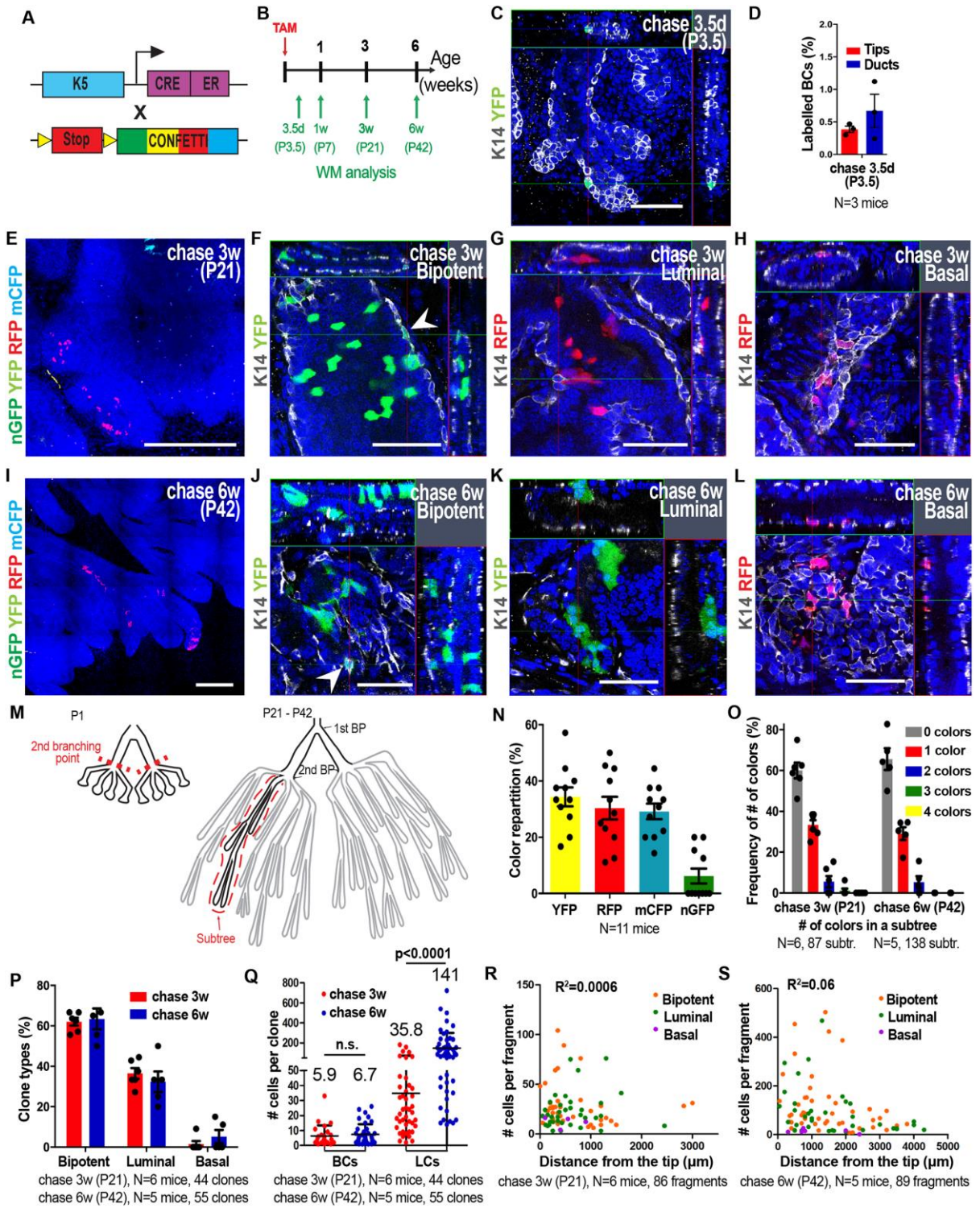


Fig. 4. Clonal analysis shows the widespread distribution of multipotent and luminal committed basal SCs at the early stage of postnatal prostate development.

(A) Genetic strategy used to label single and isolated K5 expressing BCs during early prostate postnatal development. (B) Temporal analysis of clonal lineage tracing. (C) Representative confocal image of recombined isolated BCs expressing one of the four colours of the confetti reporter 3.5 days post induction. (D) Quantification of the labelled BCs 3.5. (E) Confocal image of the VP induced at P1 and chase for 3 weeks. (F-H) Confocal images of clones derived from a single labelled BCs 3 weeks post induction. Arrowhead indicates BC. (I) Confocal image of the VP induced at P1 and chased for 6 weeks. (J-L) Confocal images of clones derived from labelled BCs 6 weeks post induction. Arrowhead indicates BC. (M) Simplified drawing displaying the evolution of the ductal tree of the ventral prostate from P1 to P21-P42. The red dashed lines show the second branching point at P1 and outlines a subtree at P21-P42 which is defined as the cluster of ducts arising after the second branching point of the prostate. (N) Recombination frequency of the different colors of the confetti reporter. (O) Frequency of observing the expression of the 4 confetti colors within a ductal subtree. (P) Quantification of clone types (bipotent, luminal and basal unipotent) 3 and 6 weeks post induction. (Q) Average basal and luminal clone sizes 3 and 6 weeks post induction. (R,S) Distribution of the number of cells per clone relative to their distance from the tips 3 weeks (R) and 6 weeks (S) post induction. In all panels K14 is visualized in grey and nuclei were counterstained with Hoechst in blue. Data show mean \pm SEM (D) and mean \pm SD (N,O,P,Q). Scale bar, 50 μ m (C,F,G,H,J,K,L) and 500 μ m (E,I). The number of clones quantified and the number of mice analysed are indicated in the respective panels. P-values were calculated by Mann-Whitney test. R² was calculate with Pearson correlation coefficient.

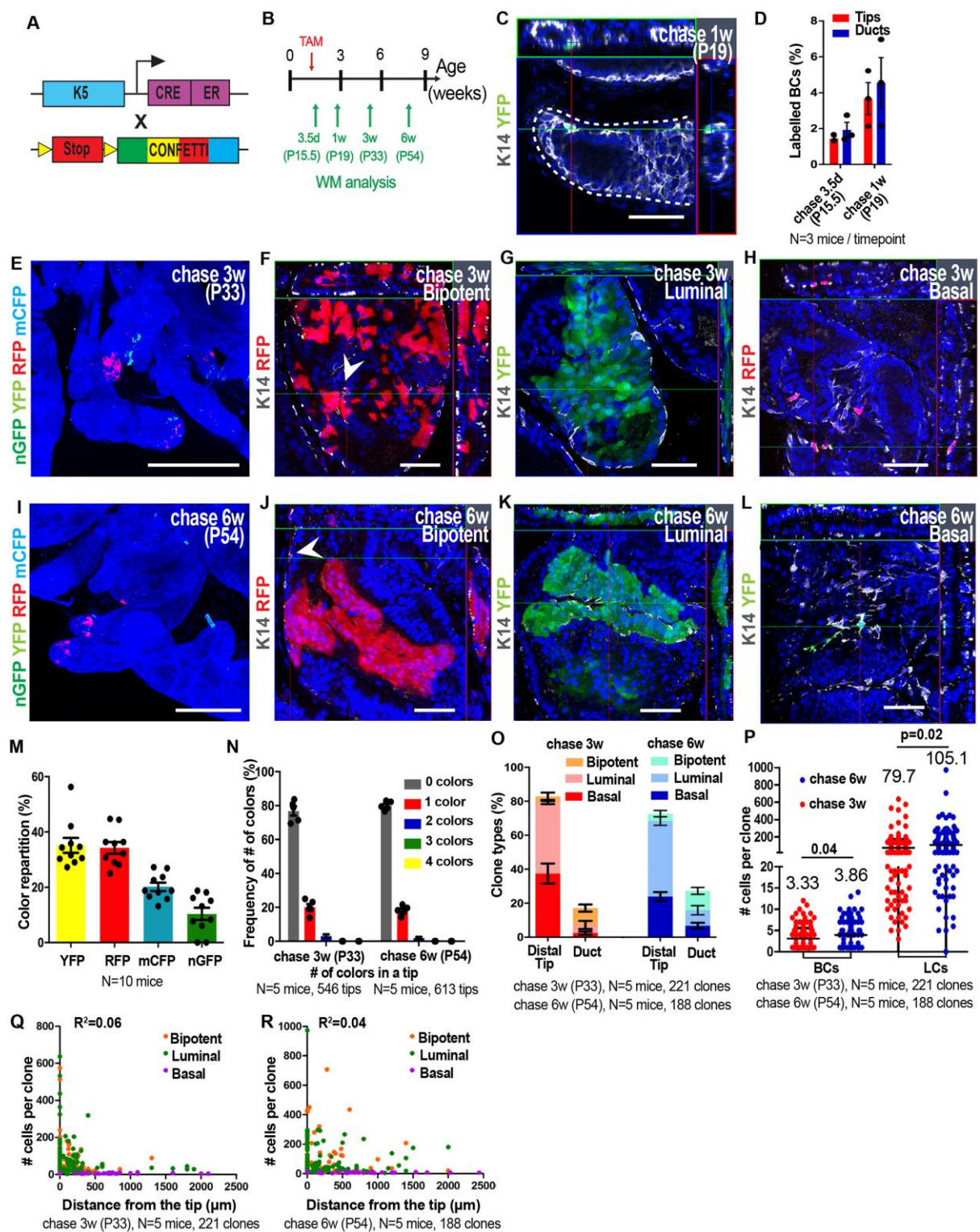


Fig. 5. Clonal analysis shows that multipotency is restricted to the tip region during mid postnatal prostate development.

(A) Genetic strategy used to label single and isolated K5 expressing BCs during mid postnatal prostate development. (B) Temporal analysis of clonal lineage tracing. (C)

Confocal image of isolated recombined BCs expressing one of the four colours of the Confetti reporter 1 week post induction. (D) Quantification of labelled BCs 3.5 and 7 days post induction. (E) Confocal image of the VP induced at P12 and chased for 3 weeks. (F-H) Confocal images of clones derived from single labelled BCs 3 weeks post induction. Arrowhead indicates BC. (I) Confocal image of the VP induced at P12 and chased for 6 weeks. (J-L) Confocal images of clones derived from single BCs 6 weeks post induction. Arrowhead indicates BC. (M) Recombination frequency of the four colours of the confetti reporter. (N) Frequency of observing the expression of the four confetti colours within a duct. (O) Quantification of clone types (bipotent, luminal and basal unipotent) according to their spatial localisation 3 and 6 weeks post induction, showing that almost all bipotent and luminal clones are found initially at the distal tip, and are progressively displaced more distally as ducts grow in length. (P) Average basal and luminal clone sizes 3 and 6 weeks post induction. (Q,R) Distribution of the number of cells per clone relative to their position from the tips 3 weeks (Q) and 6 weeks (R) post induction. In all panels K14 is visualised in grey and nuclei were counterstained with Hoechst in blue. Data show mean \pm SEM (D) and mean \pm SD (M,N,O,P). Scale bar, 50 μ m (C,F,G,H,J,K,L) and 500 μ m (E,I). The number of clones quantified and the number of mice analysed are indicated in the respective panels. P-values were calculated by Mann-Whitney test. R² was calculate with Pearson correlation coefficient.

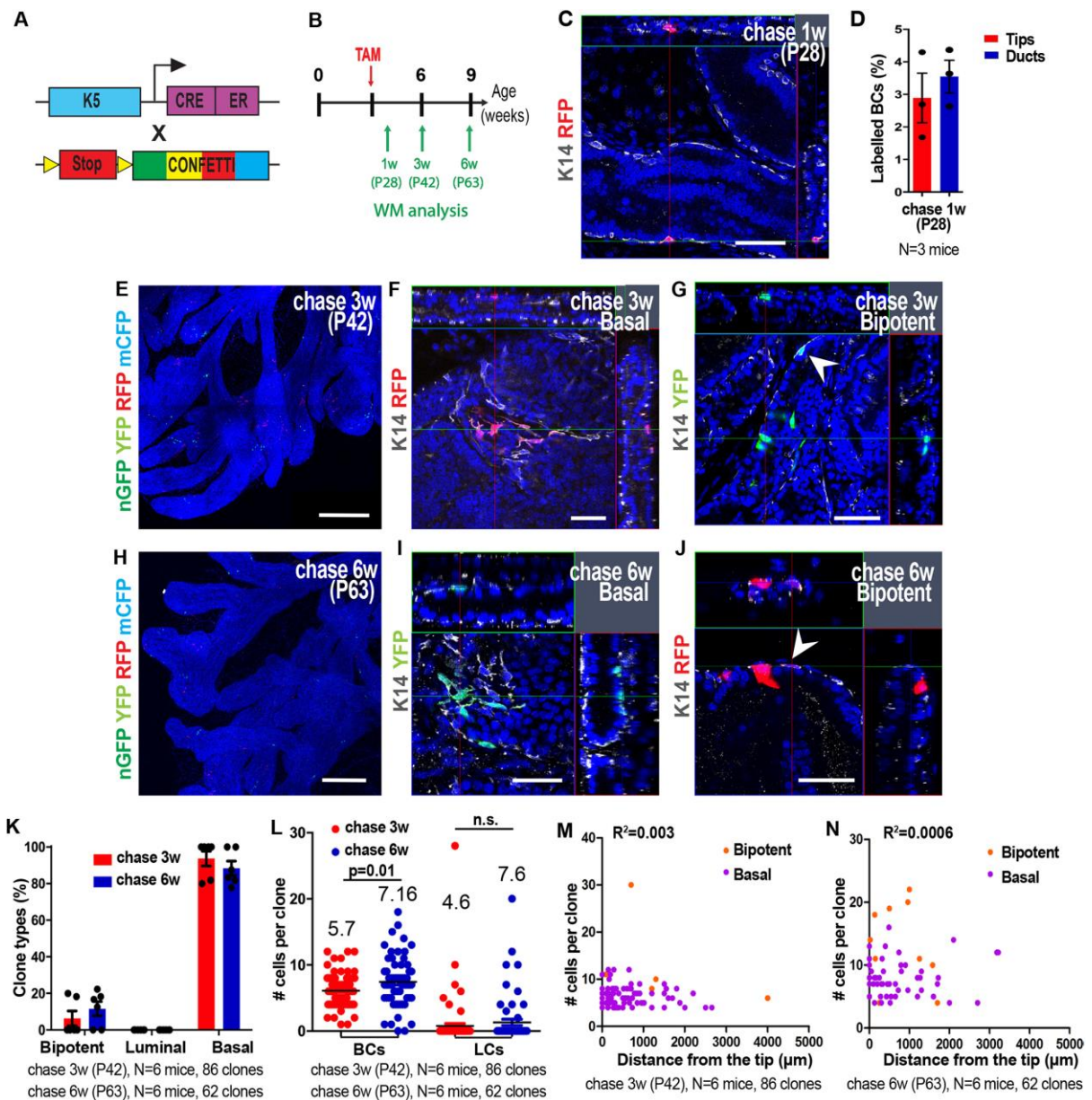


Fig. 6. Clonal analysis shows that basal stem cells become unipotent at the onset of puberty.

(A) Genetic strategy used to label single and isolated K5 expressing BCs during pubertal prostate development. (B) Temporal analysis of clonal lineage tracing. (C) Confocal image of isolated recombined BCs expressing one of the four colours of the Confetti reporter 1 week post induction. (D) Quantification of labelled BCs 1 week post induction. (E) Confocal image of the VP induced at P21 and chased for 3 weeks. (F,G) Confocal images of clones derived from single labelled BCs 3 weeks post induction. Arrowhead indicates BC. (H) Confocal image of the VP induced at

P21 and chased for 6 weeks. (I,J) Confocal images of clones derived from single BCs 6 weeks post induction. Arrowhead indicates BC. (K) Quantification of clone types (bipotent, luminal and basal unipotent) 3 and 6 weeks post induction, showing that 90% of basal cells are unipotent and less than 10% of the clones are multipotent after P21. (L) Average basal and luminal clone sizes 3 and 6 weeks post induction. (M,N) Distribution of the number of cells per clone relative to their distance from the tips 3 weeks (M) and 6 weeks (N) post induction. In all panels K14 is visualised in grey and nuclei were counterstained with Hoechst in blue. Data show mean \pm SEM (D) and mean \pm SD (K,L). Scale bar, 50 μ m (C,F,G,I,J) and 500 μ m (E,H). The number of clones quantified and the number of mice analysed are indicated in the respective panels. P-values were calculated by Mann-Whitney test. R² was calculate with Pearson correlation coefficient.

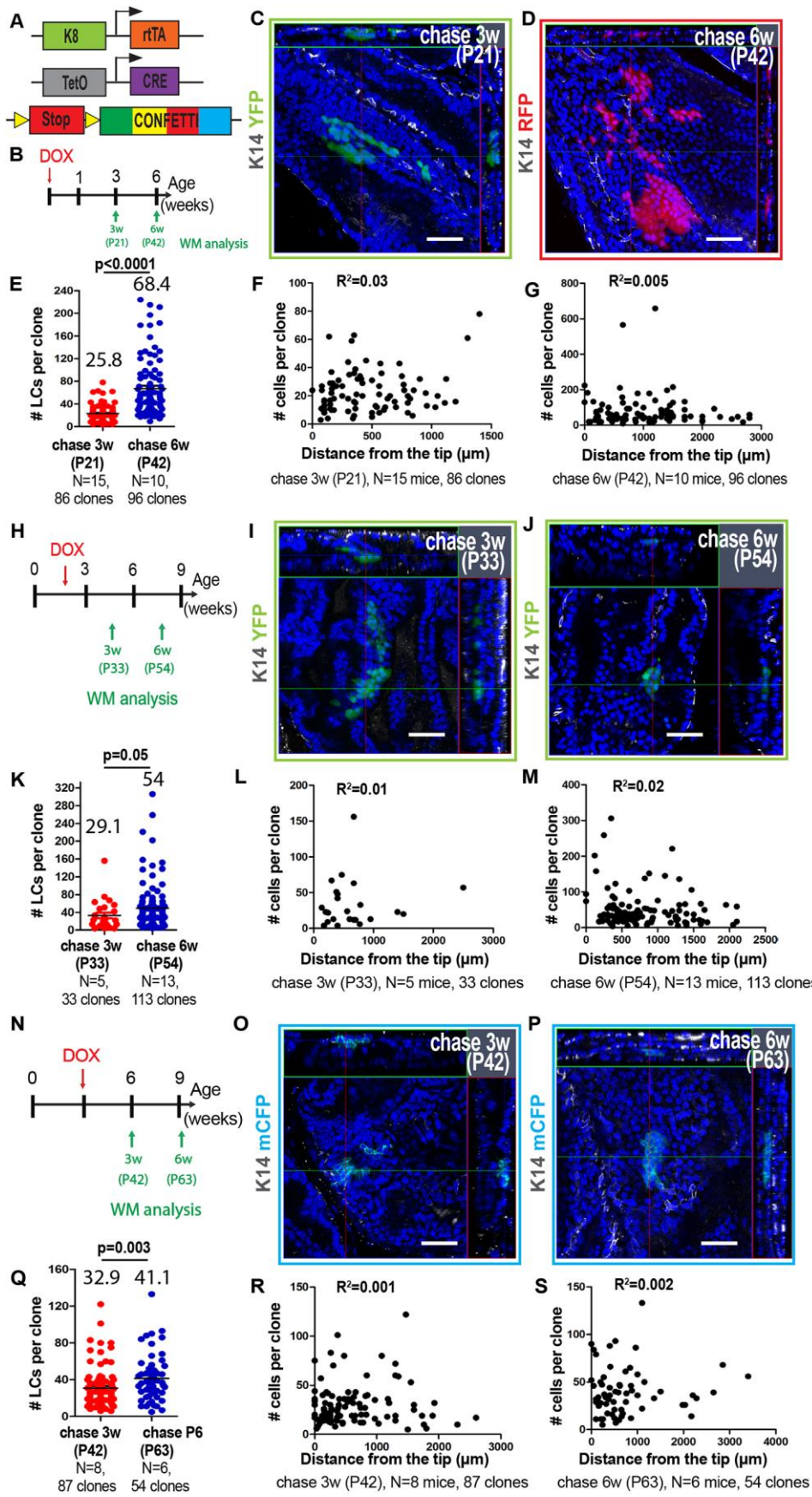


Fig. 7. Clonal analysis shows the important contribution of unipotent luminal stem cells to luminal lineage expansion during prostate development.

(A) Genetic strategy used to label K8 expressing LCs during prostate postnatal development. (B) Temporal analysis of clonal lineage tracing performed at P1. (C,D) Confocal images of clones derived from labelled LCs 3 weeks (C) and 6 weeks (D) post induction. (E) Average luminal clone sizes 3 and 6 weeks post induction. (F,G) Number of cells per clone relative their distance to the tip region from 3 (F) and 6 (G) weeks post induction at P1. (H) Temporal analysis of clonal lineage tracing performed at P12. (I,J) Confocal images of clones derived from labelled LCs 3 weeks (I) and 6 weeks (J) post induction. (K) Average luminal clone sizes 3 and 6 weeks post induction. (L,M) Number of cells per clone relative their distance to the tip region 3 (F) and 6 (G) weeks post induction. (N) Temporal analysis of clonal lineage tracing performed at the onset of puberty (P21). (O,P) Confocal images of clones derived from labelled LCs 3 weeks (O) and 6 weeks (P) post induction. (Q) Average luminal clone sizes 3 and 6 weeks post induction. (R,S) Number of cells per clone relative their distance to the tip region 3 (F) and 6 (G) weeks post induction. K14 is visualized in grey and nuclei were counterstained with Hoechst in blue. Data show mean \pm SD (E,K,Q). Scale bar, 50 μ m. The number of clones quantified and the number of mice analysed are indicated in the respective panels. P-values were calculated by Mann-Whitney test. R^2 was calculated with Pearson correlation coefficient.

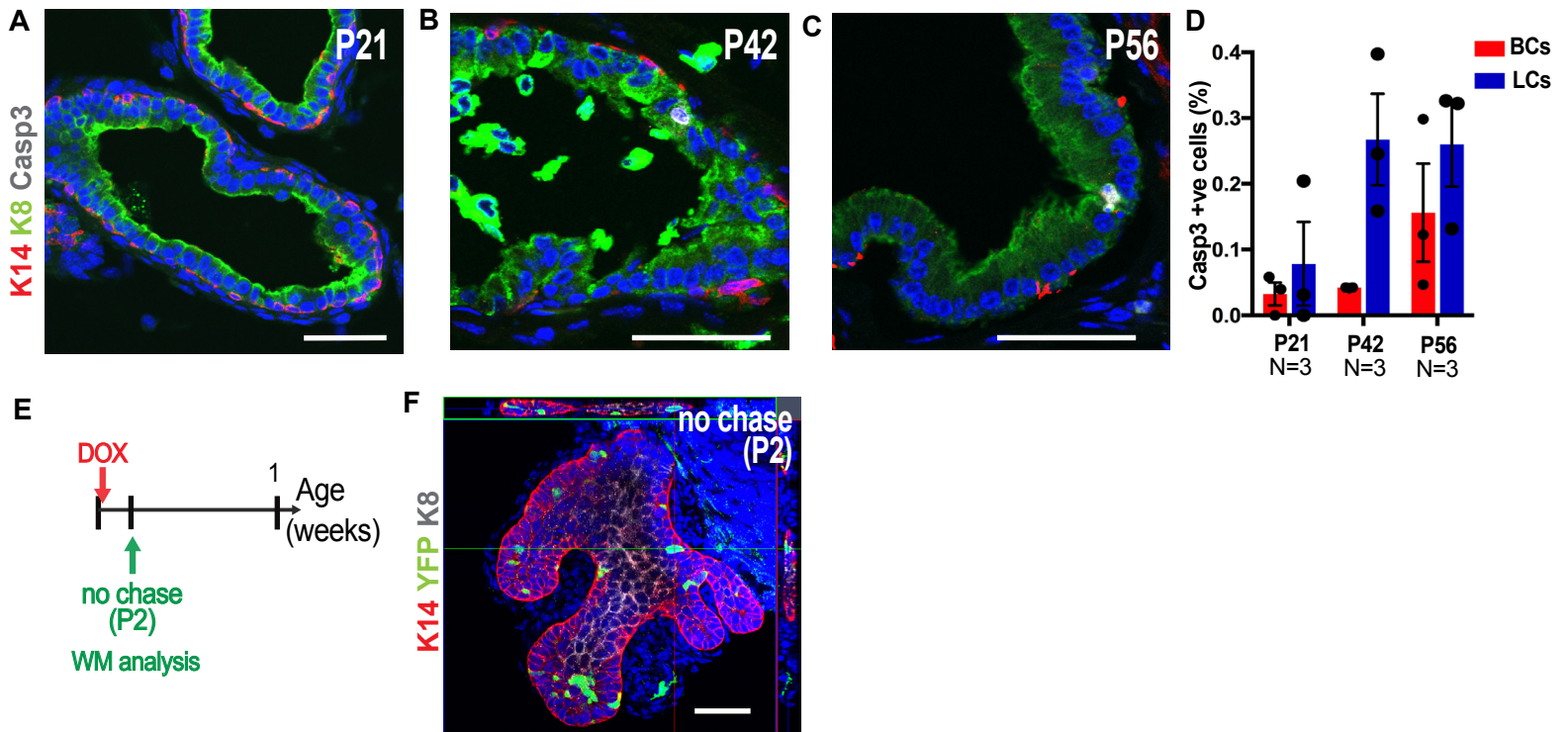


Fig. S1 (Related to Fig. 2 and Fig. 3) Analysis of Cell death and lineage tracing specificity during prostate development.

(A-C) Representative images of immunostaining of Caspase3 (grey), K14 (red) and K8 (green) on prostate sections at mice of age P21 (A), P42 (B) and P56 (C). (D) Quantification of Caspase3 positive cells at different time points during prostate development. Mean±SEM are shown from 14,016, 53,732 and 49,410 cells counted at the different time points indicated. (E) Protocol used to analyse the initial targeted cells of saturation lineage tracing at early stage of postnatal development (P1-P2). (F) Confocal image of the VP at the end of Dox administration (P2). K14 is visualized in red, YFP in green, K8 in grey. In all panels, nuclei were counterstained with Hoechst in blue. Scale bar, 50µm. The number of mice analysed are indicated in the respective panels.

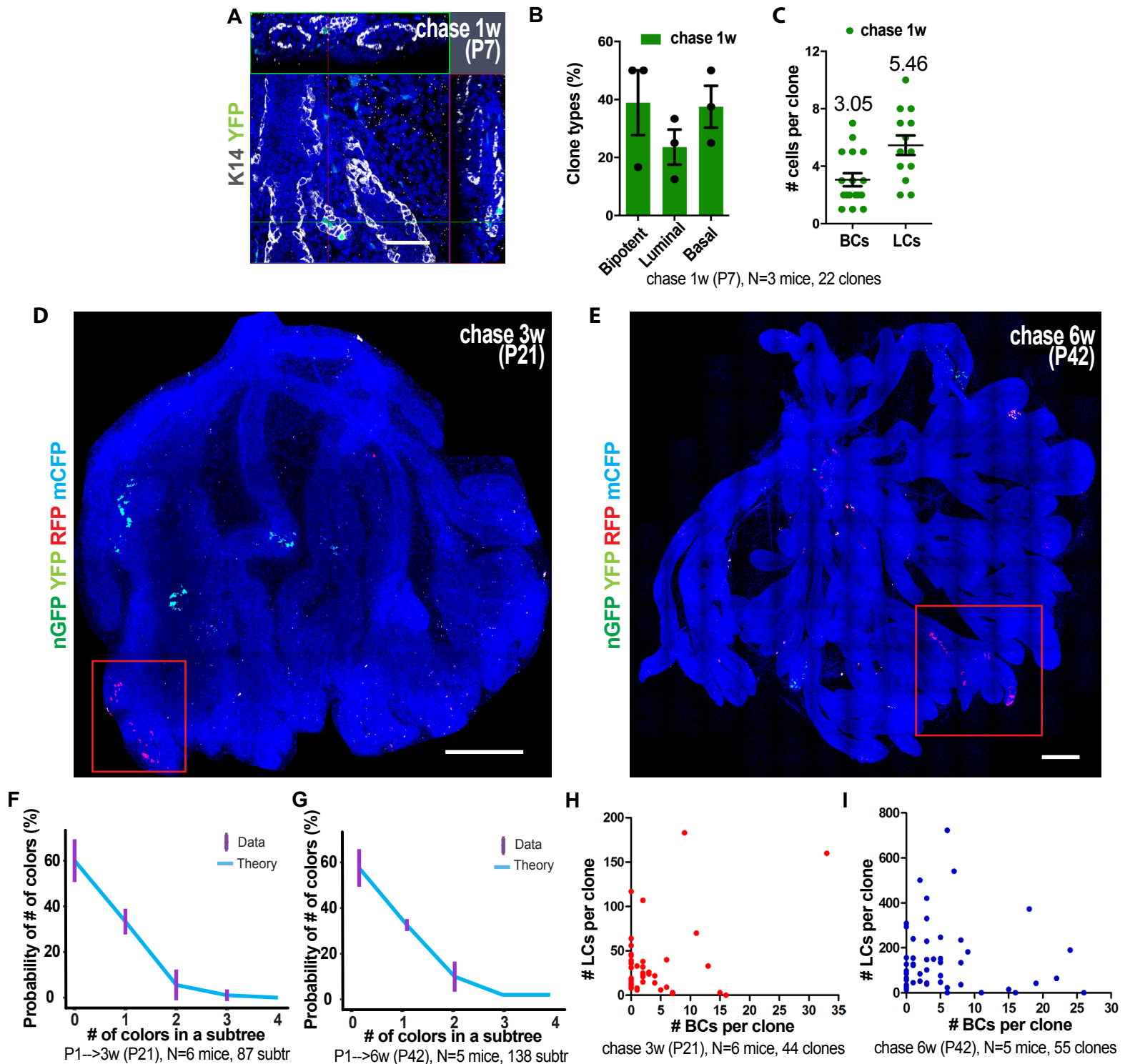


Fig. S2 (Related to Fig. 4) The contribution of multipotent basal progenitors to the prostate postnatal development analysed by confocal imaging of whole mount tissue.

(A) Representative confocal image of a clone derived from a single labeled BCs 1 week post induction. (B) Quantification of clone types (bipotent, luminal and basal unipotent) 1 week post induction. (C) Average basal and luminal clone sizes 1 week post induction. (D,E) Confocal images of the VP induced at P1 and chased for 3 weeks (D) or 6 weeks (E). Red panels are shown in higher magnification in Fig. 4E and I. (F,G) Theoretical Poisson distribution of the number of colours obtained after clonal marking in a subtree at 3 (F) and 6 (G) weeks post induction. (H,I) Number of BCs and LCs per clone derived from labeling of single BCs 3 weeks (H) and 6 weeks (I) post induction. In all panels K14 is visualized in grey and nuclei were counterstained with Hoechst in blue. Data show mean±SD (B,C). Scale bar, 50µm (A) and 500µm (D,E). The number of subtrees and clones quantified and the number of mice analysed are indicated in the respective panels.

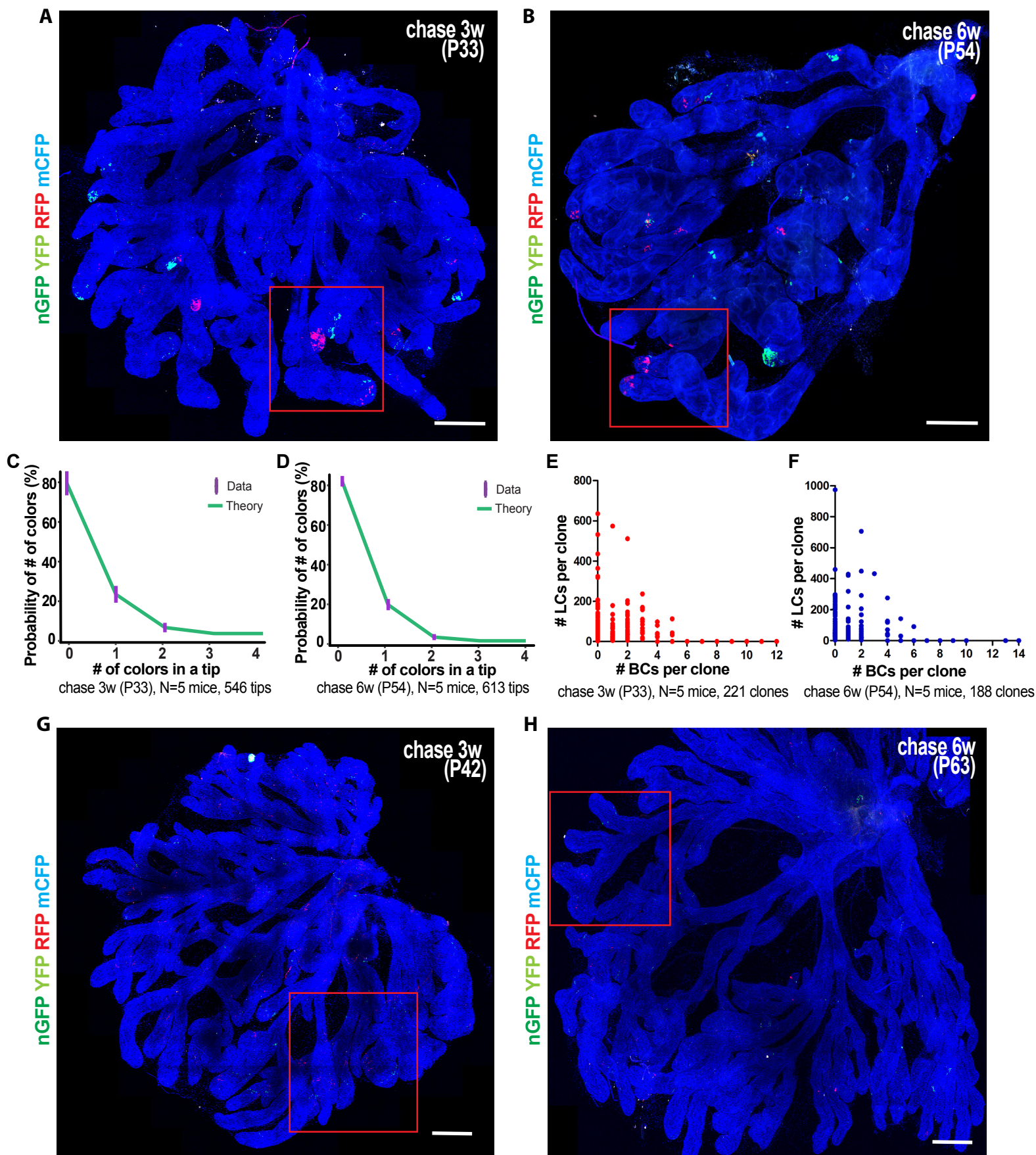


Fig. S3 (Related to Fig. 5 and Fig. 6) The spatiotemporal restriction of multipotency during prostate development.

(A,B) Confocal images of the VP induced at P12 and chase for 3 weeks (A) or 6 weeks (B). Red panels are shown in higher magnification in Fig. 5E and I. (C,D) Theoretical Poisson distribution of the number of colours obtained after clonal marking in a tip 3 (C) and 6 (D) weeks post induction. (E,F) Number of BCs and LCs per clone derived from labeling of single BCs 3 weeks (E) and 6 weeks (F) post induction. (G,H) Confocal images of the VP induced at P21 and chased for 3 weeks (G) or 6 weeks (H). Red panels are shown in higher magnification in Fig. 6E and H. In all panels nuclei were counterstained with Hoechst in blue. Scale bar, 500 μ m. The number of tips and clones quantified and the number of mice analysed are indicated in the respective panels.

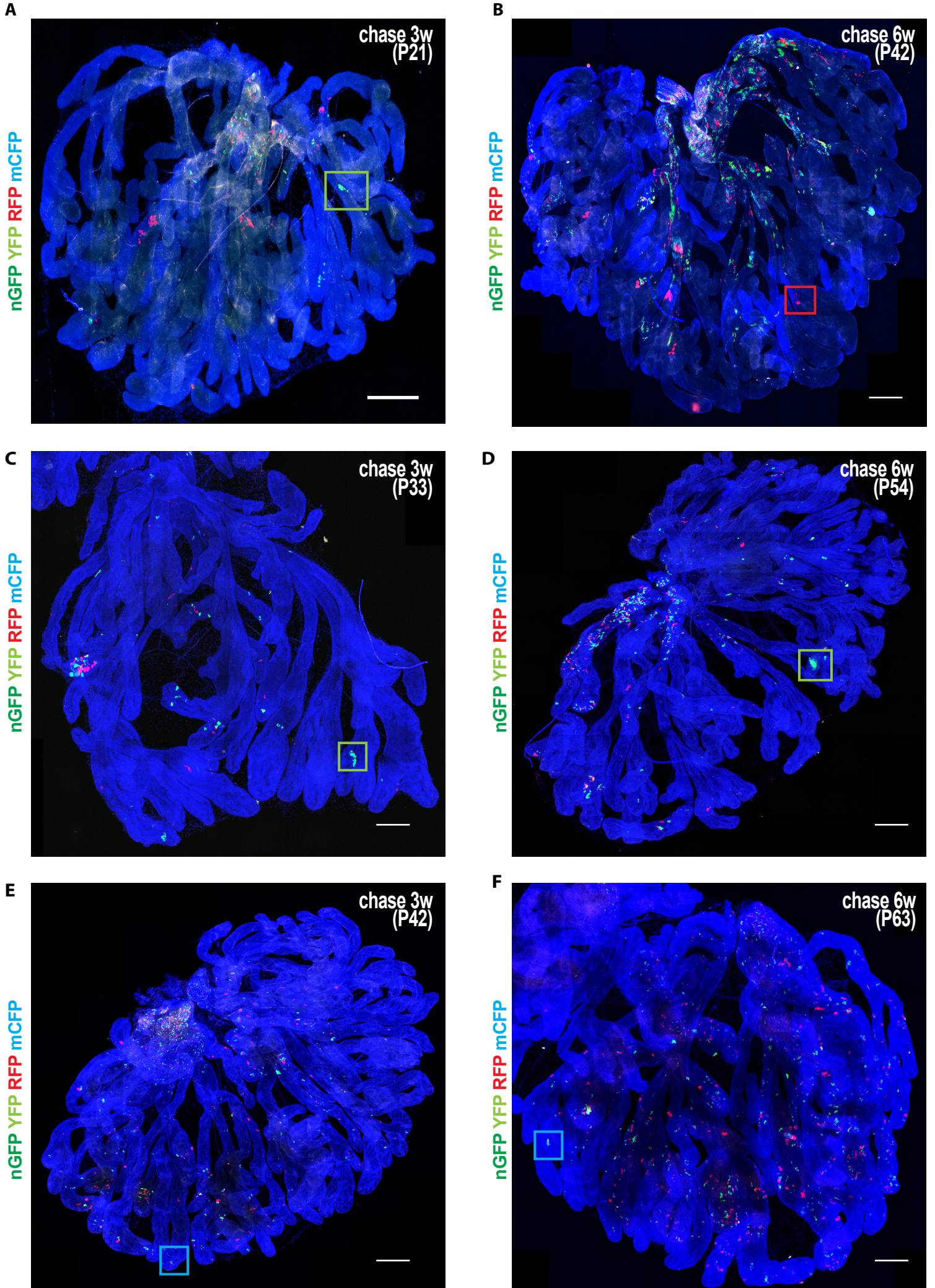


Fig. S4 (Related to Fig. 7) Confocal analysis of the fate of unipotent luminal stem and progenitor cells during prostate development.

(A,B) Confocal images of the VP induced at P1 and chase for 3 weeks (A) or 6 weeks (B). (C,D) Confocal images of the VP at P12 and chased for 3 weeks (C) or 6 weeks (D). (E,F) Confocal images of the VP induced at P21 and chased for 3 weeks (E) or 6 weeks (F). In all panels, rectangles are clones represented in higher magnification in Fig. 7, nuclei were counterstained with Hoechst in blue. Scale bar, 500 μ m.

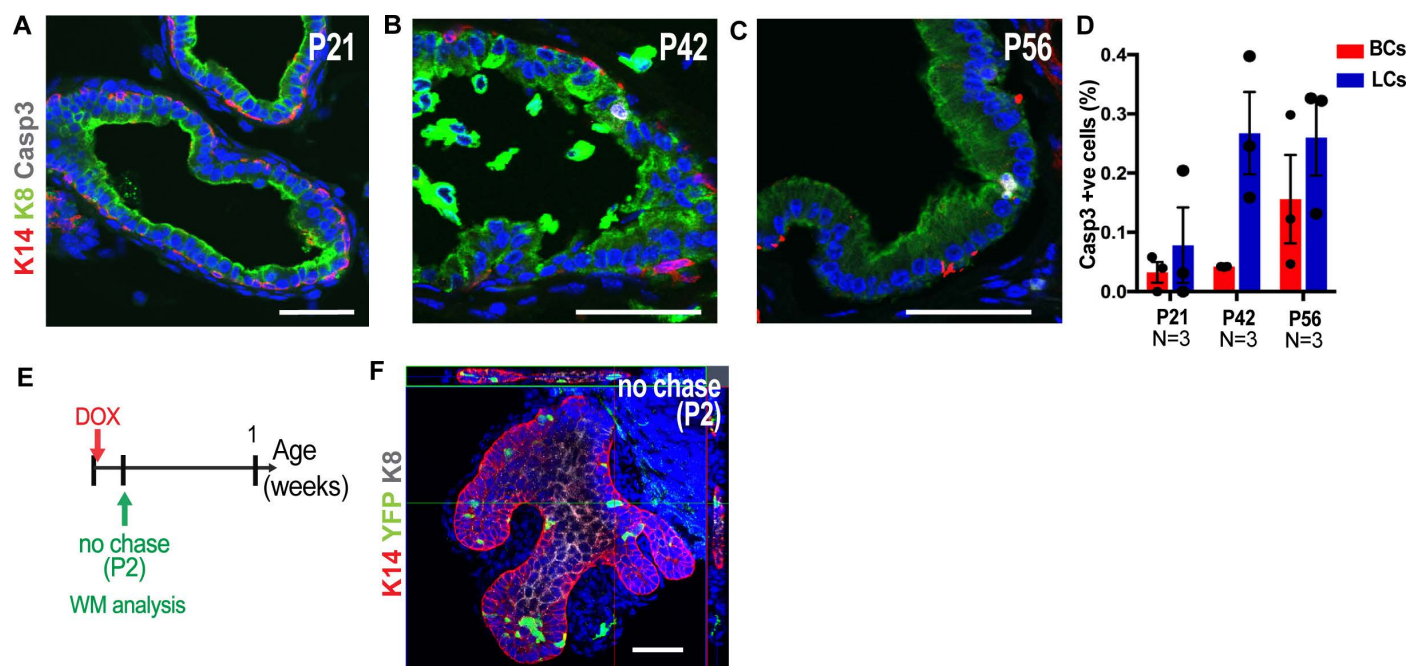


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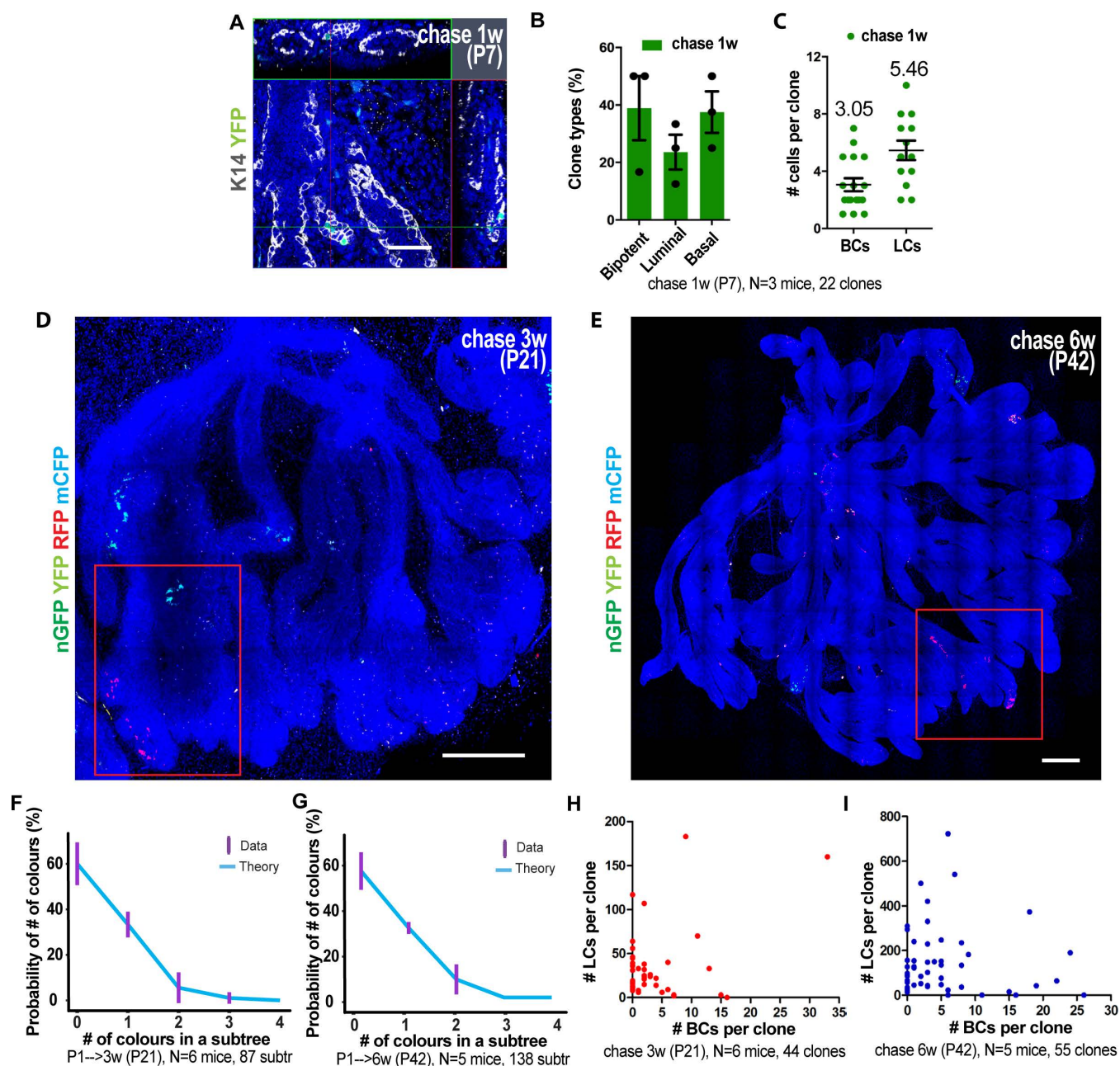


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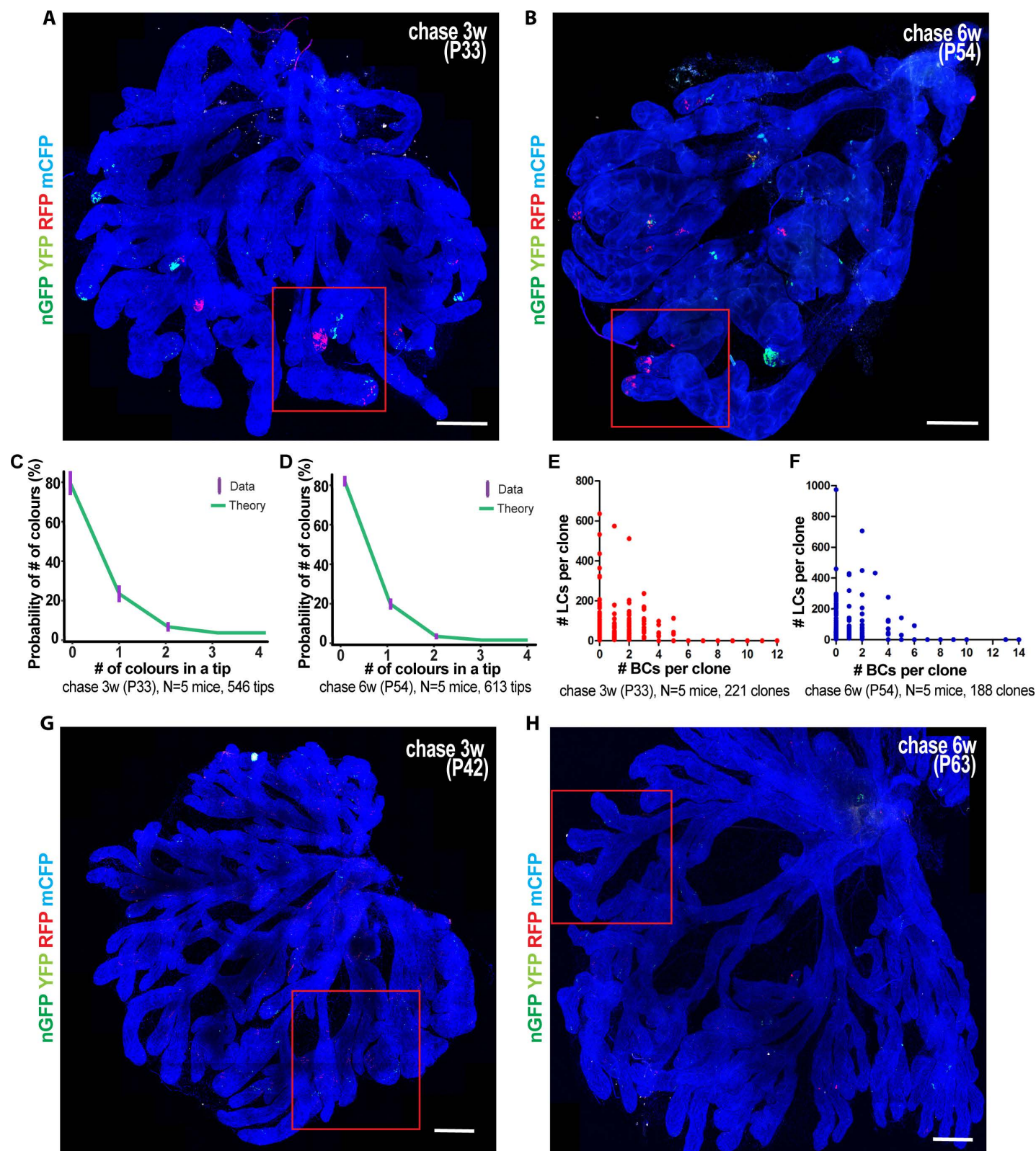


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(A,B) Confocal images of the VP induced at P12 and chased for 3 weeks (A) or 6 weeks (B). Red panels are shown in higher magnification in Fig. 5E and I. (C,D) Theoretical Poisson distribution of the number of colours obtained after clonal marking in a tip 3 (C) and 6 (D) weeks post induction. (E,F) Number of BCs and LCs per clone derived from labeling of single BCs 3 weeks (E) and 6 weeks (F) post induction. (G,H) Confocal images of the VP induced at P21 and chased for 3 weeks (G) or 6 weeks (H). Red panels are shown in higher magnification in Fig. 6E and H. In all image panels nuclei were counterstained with Hoechst 33342 in blue. Scale bar, 500 μ m. The number of tips and clones quantified and the number of mice analysed are indicated in the respective panels.

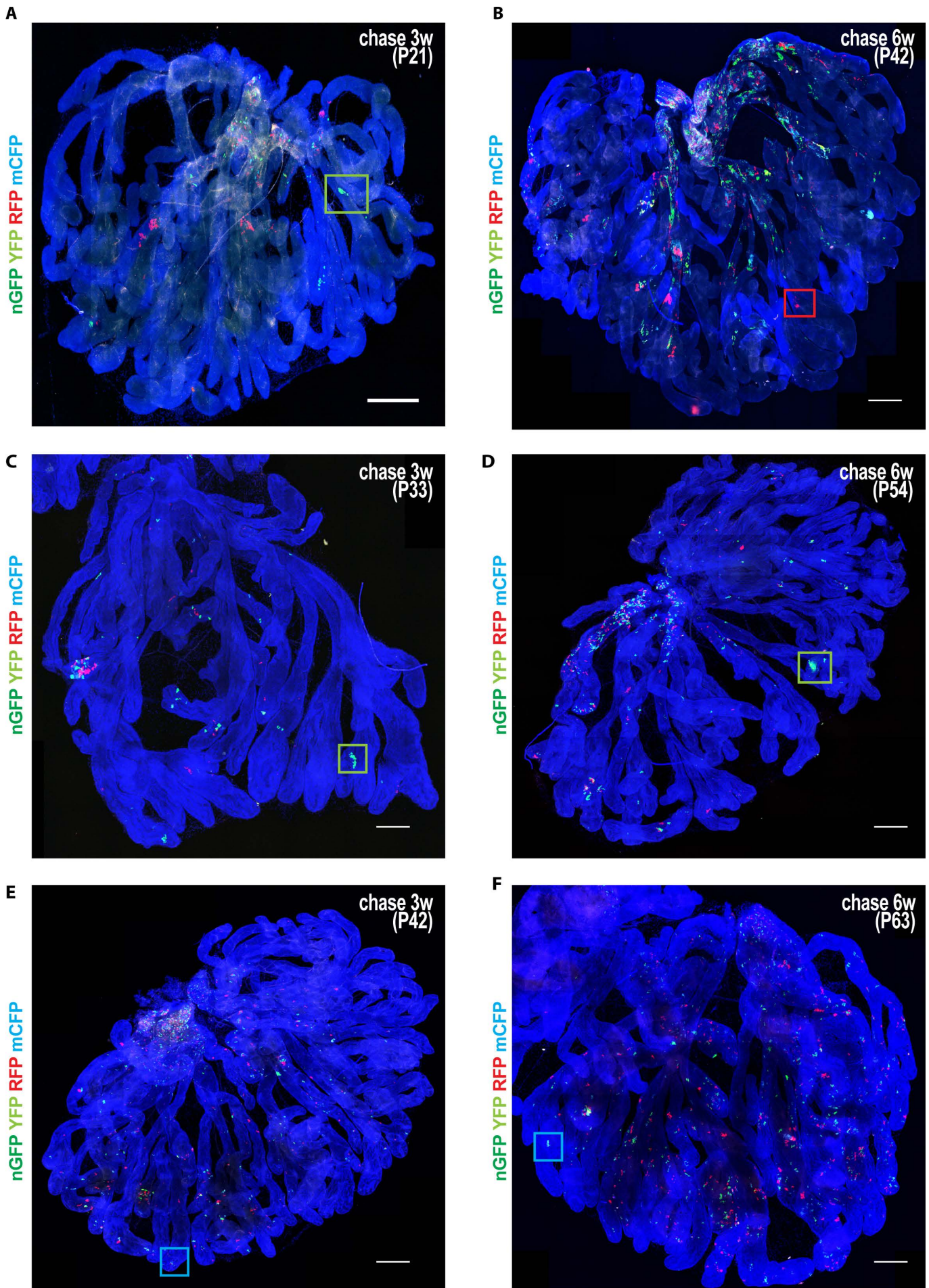


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