In the chick embryo, estrogen can induce chromosomally male ZZ left gonad epithelial cells to form an ovarian cortex which supports oogenesis

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Abstract

In chicken, as in most amniotes, the first morphogenetic event in ovary differentiation is the formation of two distinct domains: a steroidogenic core, the medulla, overlain by the germ cell niche, the cortex. This process normally starts immediately after sex determination in the ZW embryos, substantially before the progression of germ cells into meiosis. In order to address the extent to which the cortical domain depends on intrinsic or extrinsic factors, we generated models of gonadal intersex by mixing ZW (female) and ZZ (male) cells in gonad chimera, or by altering estrogen levels of ZZ and ZW embryos *in ovo*. We found that both female and male cells can become part of the cortical domain and that this can form relatively independently of the sex of the medulla as long as estrogen is provided. We also show that the cortex promoting activity of estrogen signalling is mediated via Estrogen Receptor alpha within the left gonad epithelium. Either a ZW or ZZ cortical domain thus provides an adequate niche which allows the germ cells to progress into meiosis. However, the presence of a medulla with an "intersex" or male phenotype may compromise this progression, causing cortical germ cells to remain in an immature state in the embryo. This makes meiotic entry a checkpoint for a coordinated sexual differentiation along the female pathway of cortex and medulla.

Introduction

The gonads provide a paradigm in which to investigate organogenesis given that there are two possible outcomes, ovaries and testes, which arise from a common anlagen and cell lineages.

In the chick, gonadal sex specific morphological differences become grossly apparent from around day (D) 8 (HH34) (Hamburger and Hamilton, 1951) with the re-localisation of the germ cells to the appropriate domain. In chromosomally male (ZZ) embryos the germ cells become embedded within somatic cells to form the sex cords of the gonadal core or medulla, while in chromosomally female (ZW) embryos the germ cells aggregate at the periphery of the gonad and intermingle with somatic cells to form the cortex (Carlon and Stahl, 1985). In the embryonic phase of ovary differentiation, the germ cells organise in nests. It is only after hatching, when the cortex undergoes a major restructuring, in a process known as folliculogenesis, that the nests are broken and each germ cell becomes enveloped by layer(s) of granulosa cells. The embryonic cortex is therefore transient, but it is a common feature of most amniotes, including many mammals. It precedes the entry of germ cells into meiosis. Mouse and rat are exceptions to this rule, because their germ cells remain distributed in the whole gonad long after meiotic entry (Byskov, 1986; DeFalco and Capel, 2009).

The formation of the embryonic cortex is side dependent in the chick. This is due to the interaction of the canonical left-right asymmetry pathway with the gonadal development pathway via the transcription factor *Pitx2* expressed asymmetrically in the left gonadal epithelium. During sex specific differentiation of the gonads, this asymmetry is ignored or overwritten in males, while in females it is exacerbated (Guioli and Lovell-Badge, 2007; Ishimaru et al., 2008; Rodriguez-Leon et al., 2008). Indeed, at sex determination, the medulla of both left and right embryonic ZW gonads differentiate into steroidogenic domains, but only the left gonad develops a cortex and proceeds to differentiate further into a functional ovary. The right ovary remains just as a steroidogenic organ and later atrophies. Germ cells scattered in the medulla, in both the left and right gonad, do not properly enter meiosis and are lost post hatching (Guioli et al., 2014). This asymmetry points to the gonadal epithelium as pivotal in the formation of the ovarian cortex and establishes the importance of the embryonic cortex for survival and maturation of the germ cells.

Recent chimera studies have demonstrated that somatic cells of the chick gonad medulla differentiate into either testicular cells or ovarian steroidogenic cells in a cell autonomous manner, i.e. dependent on their sex chromosome constitution (Zhao et al., 2010), however the importance of intrinsic sex chromosome identity in the formation of the cortical germ cell niche has yet to be addressed.

Like lower vertebrates and many mammals, although not the mouse, the embryonic ovaries of birds produces estrogen from the start of female specific differentiation. For chicken and some other vertebrate species, including some mammals, it has been shown that ectopic manipulation of estrogen levels at the time of sex determination can override the effects of chromosomal sex. Notably, blocking the activity of P450 aromatase in ZW embryos before sex differentiation results in female-to-male sex reversal, where both the left and right medulla start to express male markers, such as SOX9, in differentiating cords and the left epithelium fails to make a cortex. Conversely, injecting estrogen before sex differentiation in ZZ embryos results in male-to-female sex reversal. Although this phenotype is reported to revert to normal in the adult, in the embryo both medullas express FOXL2 and P450arom and a cortex develops on the left side (Akazome and Mori, 1999; Bruggeman et al., 2002; Vaillant et al., 2003; Yang et al., 2008).

During chick sex determination *Estrogen Receptor alpha* (*ER*α) is expressed in both the left and right medulla, but asymmetrically in the epithelium of the left gonad, (Andrews et al., 1997; Guioli and Lovell-Badge, 2007). This makes it a good candidate for the estrogen transducer with the hypothesis that estrogen affects the differentiation of both medulla and cortex by acting on different cell types and different pathways. Furthermore, it suggests once again the pivotal role of the epithelium in the formation of the cortex.

In order to understand the process of embryonic cortex morphogenesis, given that *ER*α RNA pattern of expression is similar in ZZ and ZW at sex determination, we investigated the importance of estrogen signalling in cortex differentiation in relation to the chromosomal sex of gonadal cells. By following the fate of mixed sex gonadal chimeras and of gonads derived from embryos with manipulated estrogen levels, we show that estrogen is the only signal necessary for cortex formation and that the cortex can be made of either ZW or ZZ cells without a bias. However an intersex phenotype may compromise the progression of the germ cells to meiosis. Finally, we show that downregulating

epithelial ERα is sufficient to severely compromise cortex formation, indicating epithelial ERα as the relevant signal transducer.

Results

Modifying estrogen levels after the point of sex determination affects cortex formation without affecting the sex identity of the medulla

In order to understand the role of estrogen in cortex differentiation and the relationship between sex specific differentiation of cortex and medulla we altered estrogen levels beyond the time when sex reversal can be achieved (Bruggeman et al., 2002). To block/reduce estrogen levels we treated D7 (HH31) ZW embryos with the aromatase inhibitor fadrozole and repeated the administration every two days (ZW-Fa embryos) (Fig. 1). Gonads recovered at D10 (HH36) showed a female medulla as expected, with no sign of masculinisation, as no male markers such as SOX9 were identified by immunostaining, similar to the ZW wildtype. However, the cortical domain of the left ovaries appeared reduced in thickness compared to controls and contained fewer germ cells (Fig. 1C). ZW left ovaries collected at D17 (HH43) were morphologically much smaller compared to ZW controls (suppl. Fig. 1), but still had a cortical domain. However, this was generally limited to the central part of the ovary (Fig. 1G).

To upregulate estrogen in ZZ embryos after the point of sex determination, we injected β-estradiol *in ovo* at D7 (HH31) (ZZ-E2 embryos) (Fig. 1). The resulting ZZ gonads collected at D10 (HH36) comprised a male medulla containing cords made of Sox9-positive somatic cells and germ cells, overlain by a narrow cortex-like domain (Fig. 1D). Left and right gonads recovered at D17 (HH43) showed a more striking phenotype, with a well-developed male medulla on both sides and a quite thick cortical domain on the left side, containing germ cell nests (Fig. 1H). Neither FOXL2 nor P450 were detected by immunostaining in the medulla of the ZZ estradiol treated gonads (data not shown). Similar results were obtained when β-estradiol was injected much later (i.e. at D9, suppl. Fig. 2).

These results show that the early differentiation of the cortex can be independent from the sex of the medulla and points to estrogen as the key inducer.

ZW or ZZ cells can contribute to the cortical domain in mixed sex gonadal chimeras

It has previously been shown that, in the chick, the medulla of the gonad differentiates in a cell autonomous manner, depending on the chromosomal sex of the component cells (Zhao et al., 2010). As the differentiating embryonic ovary is composed of two distinct

sex specific functional domains: a steroidogenic medulla and a cortical domain essential for germ cells maturation, it remains to be assessed how important chromosomal sex identity is for the formation and differentiation of a proper germ cell niche. The estrogen manipulation experiments have demonstrated that a cortex can form in a ZZ embryo, however it is not clear that this is possible under normal physiological conditions. To address this issue we generated gonadal chimeras comprised of male and female cells by transplanting D2 (HH10) lateral plate/intermediate mesoderm from the left side of donor GFP embryos into matching age and same side recipient embryos of the same or opposite sex, *in ovo*. The manipulated embryos were re-incubated and the gonads were collected at D18 (HH44) (Fig. 2).

These manipulations result in an embryo with a host derived right gonad and a left gonad containing somatic cells of host and donor origin. The germ cells, however, are always host derived on both sides, as they segregate to the germinal crescent of the embryo at stage HH5 and later migrate to the gonad via the bloodstream (Ginsburg and Eyal-Giladi, 1987; Karagenc et al., 1996; Nakamura et al., 1988).

The chimeric gonads derived from left-to-left transplants between ZW embryos (FL-FL, same-sex chimeras) were ovaries composed of a medulla and a cortex similar to those in a normal left ovary. In the donor enriched area, GFP cells contributed both epithelial and sub-epithelial somatic cells of the cortex (Fig. 2A).

Fig. 2B shows a typical chimeric left ovary from a ZW embryo containing donor cells derived from ZZ left mesoderm (ML-FL mixed-sex chimera). This ovary displays a cortex along the entire length. The area enriched in donor tissue was composed of a medulla containing host (ZW) steroidogenic cells surrounded by host (ZW) and donor (ZZ) derived (GFP positive) interstitial cells, overlain by a cortex formed by GFP donor (ZZ) cells. These areas were continuous with areas poor in donor cells, whose medulla and cortex were mainly host derived. At the junction between the two areas intermingling of host and donor cells of the cortex was evident. Transplants of ZW donor tissue into ZZ hosts (FL-ML mixed-sex chimeras) often produced chimeric ovotestes. Fig. 2C shows one such example. The ovarian portion of the chimera comprised a female medulla containing steroidogenic cells derived from the donor (GFP positive cells) as expected, overlain by a cortex containing both host (ZZ) and donor (ZW) somatic cells.

We concluded that both ZZ and ZW cells can normally contribute the somatic component of the embryonic cortex without a sex bias and therefore independently of their chromosomal sex.

Meiotic entry of cortical germ cells is compromised if the medulla is masculinised

The mitotic to meiotic switch is quite asynchronous in the chicken ovarian cortex, with many germ cells entering meiosis by D12 (HH38) and the majority initiating the synaptic process by D16 (HH42) (de Melo Bernardo et al., 2015).

We followed germ cells meiotic progression in D17 (HH43) ovaries from ZW embryos subject to late fadrozole treatment (ZW-Fa) and from ZZ embryos subject to late estradiol treatment (ZZ-E2), by analysing the expression of the double strand breaks (DSB) marker γH2AX and the synapsis marker SYCP3 (Guioli et al., 2012; Smith et al., 2008). At this stage, both markers are normally expressed in most germ cells (Fig. 3).

In fadrozole treated ZW left gonads the cortex contained germ cells positive for γH2AX and SYCP3. Similarly, in the estradiol treated ZW left gonads many germ cells expressed both markers. However in estradiol treated ZZ left gonads, which are composed of a male medulla overlain by a cortical domain made of male somatic cells and male germ cells, meiosis was compromised: some cortical germ cells expressed γH2AX but none were positive for SYCP3 as assessed by immunostaining (Fig. 3).

To investigate the importance of germ cell sex for the correct initiation of meiosis in the cortex, we generated a series of testis-to-ovary reversed ZZ embryos and a series of ovary-to-testis reversed ZW embryos, by *in ovo* treatment with estradiol or fadrozole, respectively, at D4 (HH23) (ZZ-E2(sr) and ZW-Fa(sr) embryos). From both experiments we obtained D17 embryos with incomplete gonadal sex reversal which typically showed a testis on the right and an "ovotestis" on the left. Within the latter, the inner part of the medulla was mostly male, being organised in SOX9-positive like cords structures, while the outer areas contained FOXL2 positive cells (data not shown). The medulla was generally overlain by a cortex (Fig. 3). Importantly, in ZZ-E2(sr) embryos the primordial germ cells (PGCs) are male while in ZW-Fa(sr) embryos they are female. Meiosis was compromised in both these models, as in both cases some cortical germ cells expressed γH2AX but none or very few expressed SYCP3 by immunostaining (Fig. 3).

Stra8 is a major factor involved in meiotic initiation and should be expressed in germ cells of the left cortex starting from D12 (HH38) (Smith et al., 2008). We analysed the *in situ* expression pattern of *Stra8* in the cortex of ZZ embryos exposed to late estradiol treatment. These were negative for *Stra8* at D14 (HH40) and showed only a few very faint patches at D17 (HH43), compared to the cortex of normal ZW controls and estradiol treated ZW embryos. In contrast, the D17 (HH43) cortex of ZW embryos exposed to late fadrozole treatment was positive, although at lower levels than ZW controls (Fig. 4A).

We also checked the expression of *Dmrt1*, a known key factor involved in the mitotic-meiotic switch in mouse (Matson et al., 2010; Zarkower, 2013). In chick, as in the mouse, *Dmrt1* is normally expressed in the proliferating germ cells in both differentiating ovary and testis and it is downregulated soon after meiosis starts (Guioli et al., 2014; Omotehara et al., 2014). As shown in Fig. 4B, DMRT1 is downregulated by D17 (HH43) in most germ cells of a wildtype ovary. DMRT1-positive cells were only observed at the poles of the ovary where the maturation of germ cells is delayed (de Melo Bernardo et al., 2015). In the experimental samples ZZ-E2, ZZ-E2(sr) and ZW-Fa(sr) that showed compromised SYCP3 expression, numerous germ cells throughout the cortex were strongly positive for DMRT1 (Fig. 4B).

A cortical domain contributed by ZZ male somatic cells provides a proper niche for ZW and ZZ germ cell meiotic entry

In order to assess if a cortical domain made of ZZ somatic cells can sustain the progression of the germ cells into meiosis we analysed the expression of the meiotic markers in D18 mixed-sex gonadal chimeras (Fig. 5).

In the same-sex female chimeric left ovary (FL-FL) the germ cells localised in the cortex were positive for both γH2AX and SYCP3 as expected (Fig. 5A-B). In ML-FL mixed-sex chimeric left ovary, where the germ cells are female (ZW), the pattern of cortical PGCs was similar to the FL-FL controls. This means that SYCP3 was expressed in most PGCs throughout the entire cortex, both in areas mainly contributed by host female (ZW) somatic cells and in areas mostly contributed by donor male (ZZ) somatic cells, demonstrating that male somatic cells are competent to form a proper cortical germ cells niche (Fig. 5C-D). However, in FL-ML chimeric ovotestis, where the germ cells are male (ZZ), the results were more complex. Within the ovarian portion the cortical germ cells

showed a different pattern depending on localisation. In some cortical areas many germ cells were found positive for γH2AX and SYCP3, while in other areas few or no germ cells were positive for these markers (Fig. 5E-G). The fact that their ability to enter meiosis is site dependent, indicates that male germ cell sex identity is compatible with entering the female meiotic program.

ERα downregulation in the ZW left gonad epithelium disrupts cortex differentiation

At sex determination, ERα is asymmetrically expressed in both ZW and ZZ embryos, being present in both medullas, but restricted to the epithelium of the left gonad (Andrews et al., 1997; Guioli et al., 2014). This pattern makes it a candidate estrogen signalling transducer for promoting cortical differentiation. In this study we showed that the ZZ left gonad is sensitive to estrogen beyond the initiation of male specific differentiation, implying that an estrogen transducer should be expressed asymmetrically in the differentiating testis.

We revisited the expression of ERα and performed a time-course at protein level, as the protein pattern in the male remained unclear in the previous studies. The immunostaining analysis showed a similar pattern in ZW and ZZ gonads at D7 (HH31). ERα protein was detected in the left epithelium and in some left and right medullary cells of both sexes. However the staining in ZZ gonads was generally weaker with fewer ERα cells clearly seen in the medulla. By D8 (HH34), the medullary staining was lost in males, but was retained in the left epithelium like in the female and maintained until at least D12 (HH38) (Fig. 6). This suggests that both ZW and ZZ gonads are likely to be competent to quickly respond to estrogen via ERα during sex determination. Moreover the left epithelium of the ZZ developing testis retains the potential to respond to estrogen, even though it would not normally be exposed to it at any significant level.

In order to knock down the activity of ERα in the gonadal epithelial cells, a dominant negative form of ERα, cloned into a Tet-ON plasmid for conditional gene expression (cERα524), was transfected into the left gonadal epithelium at D2.5 (HH15-17) by *in ovo* electroporation of the left dorsal coelomic epithelium (Guioli et al., 2007). The DNA was co-electroporated with other plasmids of the Tet-ON system, including T2TP for expression of the Tol2 transposase and pT2K-CAG-rTA-M2, the doxycylin-inducible activator (Watanabe et al., 2007) (Fig. 7A and M&M). cERα524 expression was induced at D4.5 (HH25) or D5.5 (HH27) and the screening was carried out at D10 (HH36) based

on the EGFP reporter expression, as a measure of the quality of transfection. Electroporation typically results in mosaic transfection which varies from sample to sample. ZW embryos displaying high levels of EGFP (e.g. Fig. 7B-C) were processed for immunostaining with markers specific to different cell types, including cortical somatic cells, medullary steroidogenic cells and germ cells. A range of phenotypes were identified within the cortex, ranging from thinning, to lack of a proper cortex along most of the ovary. The most severe phenotype was observed in ovaries exposed to doxycycline at the earliest time point. An example is shown in Fig. 7 B, E, H. In this ovary most of the medulla highlighted by P450 aromatase was overlain by a simple epithelium, with the exception of the central part, which displayed some stratification and contained a small number of germ cells (Fig. E, H). An example of a weaker phenotype is shown in Fig. 7C, F, I. The cortex was quite irregular in size and markedly thinner in areas rich in EGFP. The number of germ cells along the cortex varied accordingly to the cortex size in the electroporated samples. Although, by D10 (HH36), many EGFP cells were found in the medulla, the medullary somatic cells maintained an ovarian identity, being negative for SOX9 (data not shown) and positive for P450 (Fig. 7E-F). No other obvious phenotype was observed in the medulla. All samples still expressed *Pitx2* within the left epithelium as seen in the normal controls (Fig. 7 L-N).

To make sure that the data observed were due to specific knock down of ERα activity we performed an alternative set of experiments aiming to suppress ERα via RNAi. Supplementary Fig. 3 shows a left ovary screened at D9.5 (HH35-36) and analysed for germ cell localisation and cortical structure. As with the phenotype obtained with cERα524, the *ERα* shRNA targeted left gonad had a normal female medulla similar to the ZW wildtype control, but a severely compromised cortex in correspondence to areas enriched in EGFP cells.

Discussion

In recent years great advances have been made uncovering key sex regulators that control the commitment to the male and female programmes, mainly based on work carried out in mammals, primarily in the mouse.

However ovarian differentiation is still poorly understood. One of the main reason is that mouse ovarian morphogenetic changes, including the formation of a cortex, are much delayed to the perinatal period, making the discovery of female sex promoting factors more challenging. Moreover, the insensitivity to estrogen in the mouse embryo may reflect the evolution of a different hierarchy of early regulators, compared even to other mammals. One example of this is *Foxl2* which is critical for ovary differentiation early in development in the goat, but not until after birth in the mouse (Boulanger et al., 2014; Schmidt et al., 2004; Uda et al., 2004; Uhlenhaut et al., 2009).

The chicken ZW gonad produces estrogen and differentiates into an ovary with a clear cortex and medulla soon after the female molecular pathway initiates, making it a good model system to address early ovarian differentiation. In this study, we focused on cortical morphogenesis with the aim of understanding the differentiation of the female germ cell niche.

Estrogen signalling is an important factor in chicken female sex determination, as it can override the influence of genetic sex if perturbed at the time of sex determination (Yang et al., 2008). This holds true in lower vertebrates and some mammals including marsupials (Coveney et al., 2001; Guioli et al., 2014). However, its potential roles as anti-testis and female promoting factors are still ambiguous. To explore its action in promoting cortex formation and to understand if this process can be dissociated from female specification of the medulla, we manipulated estrogen levels after D7, so that the medulla maintained a phenotypic sex consistent with genetic sex. We found that estrogen injection in ZZ embryos generates an “intersex” gonad composed of a male medulla overlain by a female cortex, while downregulation of estrogen in the ZW embryos perturbed proper cortex formation, despite the maintenance of a female medulla. In parallel we generated a series of male:female gonadal chimeras and observed that even the presence of a small aromatase positive medullary domain was sufficient to induce cortex formation in the left gonad.

These results show that the cortex domain can, at least initially, differentiate independently from the sex of the medulla if estrogen is provided. This makes estrogen the major promoting signal for cortex development and the left epithelium a naïve tissue capable of responding to any sex environment hormonal influence.

Therefore, early cortical formation depends mainly on the presence of promoting signals, notably estrogen, normally produced in the ovarian medulla, rather than on the lack of any antagonistic signals, produced, for example, in the male medulla.

Moreover the analysis of same-sex and mixed-sex left gonadal chimera showed that under physiological conditions, ZZ and ZW cells can become part of a cortex without a sex bias and that a cortex composed of ZZ somatic cells can provide a proper niche like the female counterpart. In both cases, after an initial phase of proliferation, the germ cells entered meiosis at the appropriate time, excluding the involvement of W or Z specific genes in the control of early cortical development. It is known that *Dmrt1*, a current candidate as a male primary sex determinant in chicken (Guioli et al., 2014; Lambeth et al., 2014; Smith et al., 2009), is expressed symmetrically in the medulla, but also asymmetrically in the gonad epithelium of ZZ and ZW embryos at the time of sex determination. As *Dmrt1* is not subject to dosage compensation (McQueen and Clinton, 2009), our chimeras show that any differences in *Dmrt1* expression levels due to gene dosage between female and male somatic cells does not impact cortex formation. Therefore although the cortex is a distinct female specific domain of the ovary, which, like the male seminiferous cords counterpart, provides a niche for germ cells maturation, the somatic cells of the cortex are naïve with respect to their sex chromosomal identity.

This is different from what happens in the gonadal medulla, which differentiates in a cell autonomous manner strictly linked to the cells chromosomal sex (Zhao et al., 2010). In chicken as in the mouse, one cell type (the supporting/steroidogenic cell) is driving sex specific differentiation of the entire organ, including the cortical domain.

However, we did observe that in ZZ embryos subject to late estradiol treatment and in ovotestis commonly obtained from female to male (FL-ML) chimeras, cortical germ cells meiosis was compromised, as SYCP3, a marker of the synaptic process was absent or expressed in very few cells. Although in all these models the PGCs were genotypically male, there is evidence that this failure is not a result of the germ cell genotype. For example, although meiosis is compromised in FL-ML chimeras, there are areas with

SYCP3-positive ZZ germ cells, suggesting that the ZZ germ cells are capable of initiating meiosis in the cortex, but they do so on the basis of their localisation within the cortical domain. Moreover the left ovotestes, generated by manipulation of estrogen levels in D4 ZZ or ZW embryos, both contained cortical germ cells with compromised SYCP3 expression, therefore independently of the germ cell genotypic sex.

In chicken, as in the mouse, *Stra8* is a key factor for germ cell entry into meiosis (Bowles et al., 2016; Koubova et al., 2014; Smith et al., 2008; Yu et al., 2013). In our SYCP3 compromised models *Stra8* is almost undetectable, while DMRT1, which should be downregulated at meiosis is still present in many germ cells. This shows that at D17 these cortical domains contain germ cells that have not progressed into meiosis. The mitotic-meiotic switch is a very complex process and include many germ cell intrinsic and extrinsic factors which may act as promoters or suppressor of meiosis and are not yet properly elucidated. Our results suggest that extrinsic factors outside the cortex must be involved. It has been shown that FGF9 secreted by the Sertoli cells antagonises meiotic entry via activation of Nodal-Smad signaling in mouse spermatogonia (Tassinari et al., 2015) and in chicken ovary bFGF was found to act as a suppressor of meiosis and as a mitogenic factor (He et al., 2012). So the proximity of a male medulla in the ovotestis may antagonise meiotic entry via FGF signalling or other male driven signalling. Alternatively meiosis may be impaired by the lack or deficiency of some female inducer signals.

In humans, disorders of sexual development (DSD) are associated with an increased risk of type II germ cell cancer which are derived from two types of *in situ* neoplastic lesions: carcinoma *in situ* (CIS) or gonadoblastoma, depending on the supporting cells being Sertoli cells or granulosa cells, respectively (Hersmus et al., 2017).

The observation of mitotic and meiotic signals within CIS cells, together with the inability of these cells to ever enter meiosis, points to a dysfunctional mitotic:meiotic switch that could provide the genetic instability initiating the tumour (Cools et al., 2006; Jorgensen et al., 2013). The long standing hypothesis is that this process is initiated in fetal life, where a gonad with an intersex phenotype provides a sexually confused niche which can result in the delay or arrest of germ cell development. If these cells are not eliminated they may retain the embryonic phenotype and later transform (Jorgensen et al., 2013).

These human studies support the idea that the meiotic phenotype in our intersex chick models is due to the sexually confused environment and predict that if these germ cells are not later eliminated, they could become transformed into tumour cells. Therefore, the estrogen manipulated chick models might even provide an important system in which to study the dynamics of neoplastic transformation of germ cells from its origin.

On the basis on the RNA expression pattern, which is symmetric in the medulla and asymmetric in the left epithelium of both male and female we have previously proposed that estrogen signalling could be transduced at sex determination via ERα. The protein expression pattern in the female was shown to reflect the RNA pattern while in the male remained elusive (Andrews et al., 1997; Guioli and Lovell-Badge, 2007). The timecourse analysis described here clearly shows that the protein is produced in the left epithelium of both ZW and ZZ gonads before and during sex specific differentiation. This demonstrates that the left epithelium of both sexes is sensitive to estrogen signalling and explains the ability to rapidly "feminise" the male left gonad even several days after sex determination.

In agreement with predictions, the knock down of ERα in the epithelial cells of the left gonad has affected cortical development. Moreover similar results were obtained using two independent approaches: a dominant negative form of ERα, or RNAi, indicating that the defects were due specifically to downregulation of epithelial ERα. In both types of experiments, the left gonad of D9-10 ZW embryos displayed a normal female medulla expressing female specific markers comparable to the ZW wild type, overlain by a compromised cortical domain, characterised by different degrees of thinning. The most severe cases displayed areas devoid of a cortex and overlain by a simple epithelium, a phenotype at least as severe as that observed in the D10 embryos treated with fadrozole from D7. This indicates ERα as the main transducer of Estrogen signalling in cortex formation and that this process strictly depends on its activity within the left epithelium.

In summary these data suggest a model whereby the gonads, at the time of somatic sex specific differentiation, are formed of two distinct domains: the epithelium and the medulla. Left and right medulla differentiates along the male or female pathway depending on whether the cells comprising it are ZW or ZZ. On the other hand the epithelial and sub-epithelial cells of the cortex can be ZW or ZZ, with no intrinsic sex bias; the cells are naïve with respect to their chromosome sex identity.

Estrogen, normally provided by the female medulla, induces and maintains the formation of the cortex via activation of ERα within a sexually naïve epithelium. That this activation can also occur above a male medulla, shows that estrogen is sufficient for this activation and suggests that the initial formation of the cortex relies on positive inductive signals more than the lack of antagonising signals.

Later progression of the cortical germ cells into meiosis requires a medulla of the correct phenotypic sex, suggesting that meiotic entry is a checkpoint of a well-coordinated ovarian cortex/medulla differentiation pathway.

Our results provide new insights into the process of chicken ovarian differentiation and provide a model that could be extended to other systems, including many mammals. Moreover, the chick intersex models may be a potential valuable system for investigating the aetiology of germ cell tumours.

Material and Methods

Animals

Most experiments were performed using Dekalb white chicken eggs obtained from Henry Stewart, Louth, UK, except for the generation of chimeras, done using GFP transgenic chickens (Roslin greens) and ISA brown chickens held at the National Avian Research Facility (NARF) at Roslin Institute, Edinburgh, UK.

Plasmids and *in ovo* electroporation

The Tol2 conditional integration plasmid system was a kind gift of Yoshiko Takahashi (Kyoto, Japan) and includes: the transposase expression plasmid pCAGGS-T2TP, the conditional expression plasmid PT2K-B1-TRE-EGFP (carrying an EGFP reporter), the Tet-ON activator plasmid pT2K-CAG-rTA-M2 which responds to doxycycline (Watanabe et al., 2007). A truncated isoform of ERα lacking the last 196bp of the open reading frame (65aminoacids) was generated by PCR using primers F-5'ACTAGTTCTAGAGCCATTAGCAATGACCATGAC and R-5'GTCGACTCTAGATTAACACTTCATATTGTACAGGTG, cloned in pCRII-TOPO (Invitrogen) and then moved to PT2K-B1-TRE-EGFP (using BamHI and EcoRV sites) to make cERα524. This isoform corresponds to a human ERα isoform shown to have a powerful dominant negative activity (Ince et al., 1993).

Three *ERα* shRNA molecules were cloned downstream of a mouse U6 promoter into pCRIITOPO using a PCR based approach aiming to amplify the U6 promoter along with the shRNA, similar to the approach described in (Harper et al., 2005). Common forward primer: U6-Forward, 5'TCTAGATCGACGCCGCCATCTCTAG; U6-reverse-shRNA specific primers:

(ER1)CTCGAGAAAAAAGGAACACCCAGGAAAGCTTTCCCATCTGTGGCTTTA
CAGAAAGCTTTCCTGGGTGTTCCAAACAAGGCTTTTCTCCAAGGG;

(ER2)CTCGAGAAAAAAGCCACTAACCAGTGTACTATCCCATCTGTGGCTTTA
CAGATAGTACACTGGTTAGTGGCAAACAAGGCTTTTCTCCAAGGG,

(ER3)CTCGAGAAAAAAGGTACCCTACTACCTTGAAACCCATCTGTGGCTTTA
CAGTTTCAAGGTAGTAGGGTACCAAACAAGGCTTTTCTCClAAGGG.

The sequences include restriction enzymes for cloning. ER1 and ER2 showed high silencing activity in luciferase assays (see below) and were selected for the experiments. The resulting U6-shER fragments (ER1 and ER2) were cloned into pSLAX13 previously

modified to carry a IRES-EGFP fragment. U6-shER-IRES-EGFP (ER1 and ER2) were then moved to a RCAS(A) retroviral vector to generate RCAS-U6-shER-IRES-EGFP (ER1 and ER2).

Dominant negative activity of cERα524 and silencing activity of the three shRNA guides were assessed by luciferase assay in vitro, following transfection in HEK293 cells together with full length cERα (cloned into pSG1) and the 3XERE-TATA-Luc luciferase reporter (Lemmen et al., 2004), kind gift of dr. Paul T. van der Saag, Ubrecht Institute, Utrecht, Netherlands (data not shown). *In ovo* electroporation of plasmid DNA into the left gonadal epithelium was achieved by DNA injection into the left coelomic cavity of HH15-17 embryos using a glass capillary needle and an Inject+matic pico pump, followed by electroporation using a NEPA21 electroporator (Sonidel) (five 50ms pulses at 26V, transferring pulse only). Detailed procedure as previously described (Guioli et al., 2007).

In ovo drug treatment

For manipulation of estrogen levels after sex determination (late treatment) eggs were incubated at 37.7°C pointed end down. At D7 or D9 a first injection in the air chamber was performed through a hole made at the rounded end. To downregulate estrogen levels the P450aromatase inhibitor Fadrozole (Sigma F3806) was injected at 0.5mg/egg in 50ul of PBS and then re-injected at 0.3mg/egg every other day until ready for analysis. To increase estrogen levels ß-Estradiol (Sigma E2758) was injected at 120μg/egg in 25μl 95% ETOH. Embryos collected after D14 were injected once more at D13. For manipulation of estrogen levels before sex determination (early treatment) eggs were injected once at D4 with 0.5mg or 1mg/egg of Fadrozole, or 140μg/egg of ß-estradiol.

Generation of gonadal chimera

HH10/12 (13–15 somites) GFP transgenic embryos (Roslin greens) and ISA brown embryos were used as donor and host, respectively. Details of the procedure are described in (Zhao et al., 2010). The embryos were incubated until D18.

Antibodies, Immunohistochemistry and in situ hybridisation

The following antibodies were used: rat anti-VASA (1:1000) (Aramaki et al., 2009), rat anti ERα (1:200, Fitzgerald), rabbit anti-DMRT1 (1:1500) (Guioli and Lovell-Badge,

2007), goat anti-SOX9 (1:500, R&D), goat anti-FOXL2 (1:400, Abcam), mouse anti-P450aromatase (1:200, Serotec), mouse anti-γH2AX (1:200, Upstate), rabbit anti-SYCP3 (1:500, Novus), mouse anti-Fibronectin (1:100, Developmental Studies Hybridoma Bank), goat anti-LHX9 (1:200, Santacruz). The *Stra8* probe template for RNA *in situ* was generated by PCR cloning into PCRIITOPO (Invitrogen), using primers F-5'TACCCAGACACCTCATCCCC and R5' TCAAAGGTCTCCGTGCACCG. The *Pitx2* probe was previously described in (Logan et al., 1998). Urogenital ridges were fixed in 4% paraformaldehyde at 4°C overnight, rinsed in PBS at RT, then transferred to 30% sucrose overnight and finally embedded in OCT and stored at -80°C. Cryosections for immunofluorescence were rinsed 3X five minutes in PBS and transferred for 1 hour to a blocking solution (PBS/0.1% triton, 2% donkey serum) before adding the primary antibody ON at 4°C (or 37°C for ERα). After 3X ten minute washes in PBS/0.1%tween, the sections were incubated with secondary antibodies (Invitrogen Alexa fluor-conjugated donkey antibodies 1:400 in PBS/0.1% tween). The results from the *in situ* hybridization on cryosections were imaged using a Leica DM-RA2 upright microscope equipped with a Retiga 200R camera and Q-capture Pro7 software (Q imaging). Fluorescence images were collected on a Olympus VS120 slide scanner equipped with a XM10 monochrome camera and a VS-ASW-L100 software (Olympus), or on a Leica upright SPE confocal system.

Figure legends

Fig. 1 Perturbing estrogen levels at embryonic D7 affects cortex formation in ZW and ZZ embryos. Sections from left gonads at D10 (A-D) or D17 (E-H) double-stained for the Sertoli marker SOX9 (red) and a germ cell marker: VASA or P63 (green). (A, E) ZW controls; (B, F) ZZ controls; (C, G) ZW gonads treated with Fadrozole (Fa); (D, H) ZZ gonads treated with ß-estradiol (E2). Decreasing estrogen in ZW embryos after sex determination compromise the development of the cortex; adding ß-estradiol in ZZ embryos after sex determination induces the formation of a cortex on top of the male medulla.

Fig. 2 ZW and ZZ cells contribution to the somatic component of the cortex domain. Gonadal chimeras generated by transplanting posterior lateral plate/intermediate mesoderm from donor GFP embryos (green) into recipient wildtype embryos at stage HH10. Gonads were dissected at D18 (HH44), sectioned and double-stained for the germ cell marker VASA (blue) and P450aromatase (red). In each panel (A-C) from left to right: schematic of the transplant; whole-mount image of the resulting gonads dissected at D18 (HH44); low magnification images of immunostained sections; high magnification of details from the staining corresponding to the areas highlighted in orange boxes. (A) Female-Left (FL green) to female-Left (FL) chimera: the chimeric gonad is an ovary and the GFP donor cells contribute to all somatic cell types as expected. (B) Male-Left (ML green) to Female-Left (FL) chimera: the gonad is an ovary and the green donor male cells contribute to both epithelial and sub-epithelial somatic cells of the cortex. (C) Female-Left (FL green) to Male-Left (ML) chimera: the gonad is an ovotestis. The GFP enriched portion has formed an ovarian domain with a cortex whose somatic cells are contributed by green donor female cells and male host cells.
White dashed lines define the chimeric domain, white dotted lines define the exclusively host domain. White dashed-dotted lines define the cortex-medulla border.

Fig. 3 Expression of germ cells meiotic markers in embryos subject to estrogen levels alterations. Sections from left gonads at embryonic D17 (HH43) immunostained for γH2AX (green) or SYCP3 (red). ZW-WT: ZW wildtype gonad. ZW-Fa: ZW gonad treated with fadrozole at D7; ZW-E2: ZW gonad treated with ß-estradiol at D7; ZZ-E2:

ZZ gonad treated with ß-estradiol at D7; ZW-Fa(sr): ZW gonad treated with Fadrozole at D4 (partially sex reversed); ZZ-E2(sr): ZZ gonad treated with Fadrozole at D4 (partially sex reversed). In ZW-Fa and ZW-E2 many germ cells express SYCP3, although SYCP3 appears to be less widespread in ZW-Fa than ZW control. In ZZ-E2, ZW-Fa(sr) and ZZ-E2(sr)) some germ cells express γH2AX but none clearly expresses SYCP3.

Fig. 4 Expression pattern of mitotic-meiotic switch markers in embryos subject to estrogen levels alteration. (A) RNA *in situ* analysis of *Stra8* expression on left gonad sections from embryos at D14 and D17. ZW: female wildtype control; ZZ-E2: ZZ, ß-estradiol treated at D7; ZW-E2: ZW, ß-estradiol treated at D7; ZW-Fa: ZW, Fadrozole treated at D7. *Stra8* expression is severely compromised in the ZZ gonads from embryos exposed to ß-estradiol.
(B) Immunofluorescent detection of DMRT1 (red), LHX9 (green), and fibronectin (blue) on cryostat sections of gonads from D17 embryos. LHX9 and Fibronectin mark the somatic cells of the cortex and the cortex-medulla border, respectively (Guioli and Lovell-Badge, 2007). (a) ZW: female wildtype control, boxed area enlarged in (b, c); (d) ZZ-E2: ZZ, treated with ß-estradiol at D7, boxed area enlarged in (e, f); (g) ZZ-E2(sr): ZZ, treated with ß-estradiol at D4 (partially sex reversed), boxed area enlarged in (h, i); ZW-Fa(sr): ZW, treated with fadrozole at D4 (partially sex reversed), boxed area enlarged in (k, l). In the control female DMRT1 is only present in germ cells at the gonad poles. In ZZ-E2, ZZ-E2(sr) and ZW-Fa(sr) DMRT1 is expressed in many germ cells across the cortex.

Fig. 5 Expression of meiotic markers in germ cells in the ovarian cortex of D18 chimeras. Sections from chimeric left gonads at D18 immunostained for γH2AX (blue) and SYCP3 (red). Donor cells are GFP positive (green). (A) FL-FL control; (C) ML-FL mixed-sex chimera (E) FL-ML mixed sex chimera; (B), (D) and (F, G) are high magnification of orange dashed boxed areas in (A), (C) and (E) respectively. In FL-FL and in ML-FL chimeras most cortical germ cells express SYCP3. In the FL-ML chimeric ovotestis, SYCP3 is expressed only in some cortical germ cells located in particular areas. White dashed lines highlight the portion of the chimera enriched in donor somatic cells, white dotted lines highlight the portion mainly contributed by the host somatic cells.

White dashed-dotted lines define the cortex-medulla border. In (B), (D), (F), (G) GFP has been omitted for better visualisation of the meiotic markers pattern.

Fig. 6 ERα expression time-course. Sections from ZW and ZZ left and right gonads between embryonic D7 (HH31) and D12 (HH38) immunostained for ERα (red). ERα is expressed in both ZW female and ZZ male left gonadal epithelium during sex differentiation. Dotted lines in left gonads highlight the cortex-medulla border.

Fig. 7 Suppression of epithelial ERα activity by *in ovo* electroporation of a dominant negative isoform. (A) Schematic of the inducible TET-ON plasmid system used to express a dominant/negative isoform of ERα (dnERα) in the gonad epithelium. (B-C) Whole mount images of two ZW left gonads electroporated at D2.5 (HH15-17) and screened at D10 (HH36). Targeted cells are highlighted by expression of a GFP reporter (green). (D-I) Fluorescence images of gonad sections immunostained for P450aromatase (P450) (red) or the germ cell marker VASA (red); (D, G) ZW control; (E, H) and (F, I) ZW electroporated gonads shown in (B) and (C), respectively. The cortex is severely compromised by downregulation of ERα activity. (L-N) *Pitx2* RNA in situ expression pattern: (L) ZW control, (M) and (N) ZW electroporated gonads shown in (B) and (C), respectively. *Pitx2* expression is not affected by downregulation of ERα in the left epithelium. Dotted lines mark the cortex/medulla border.

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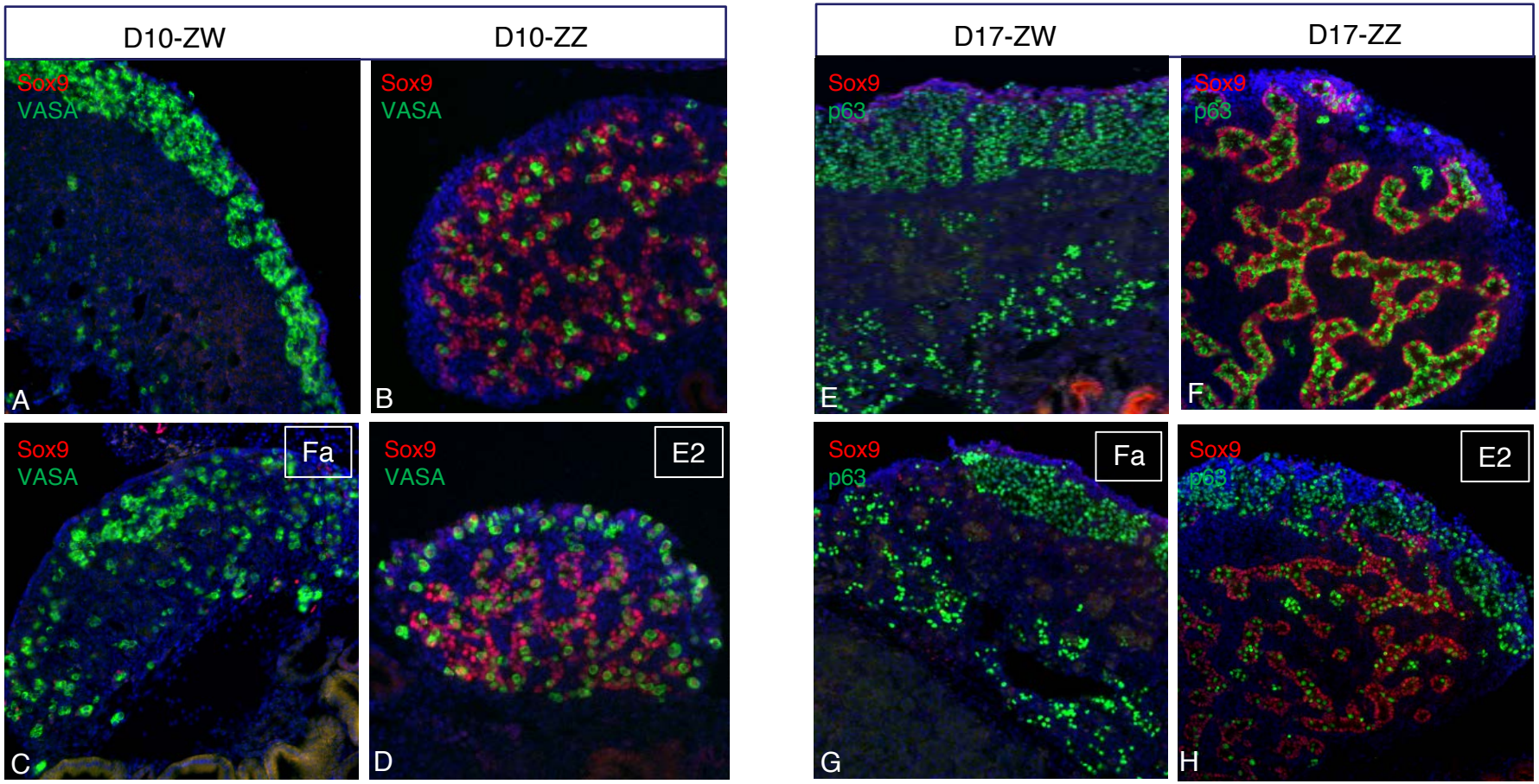
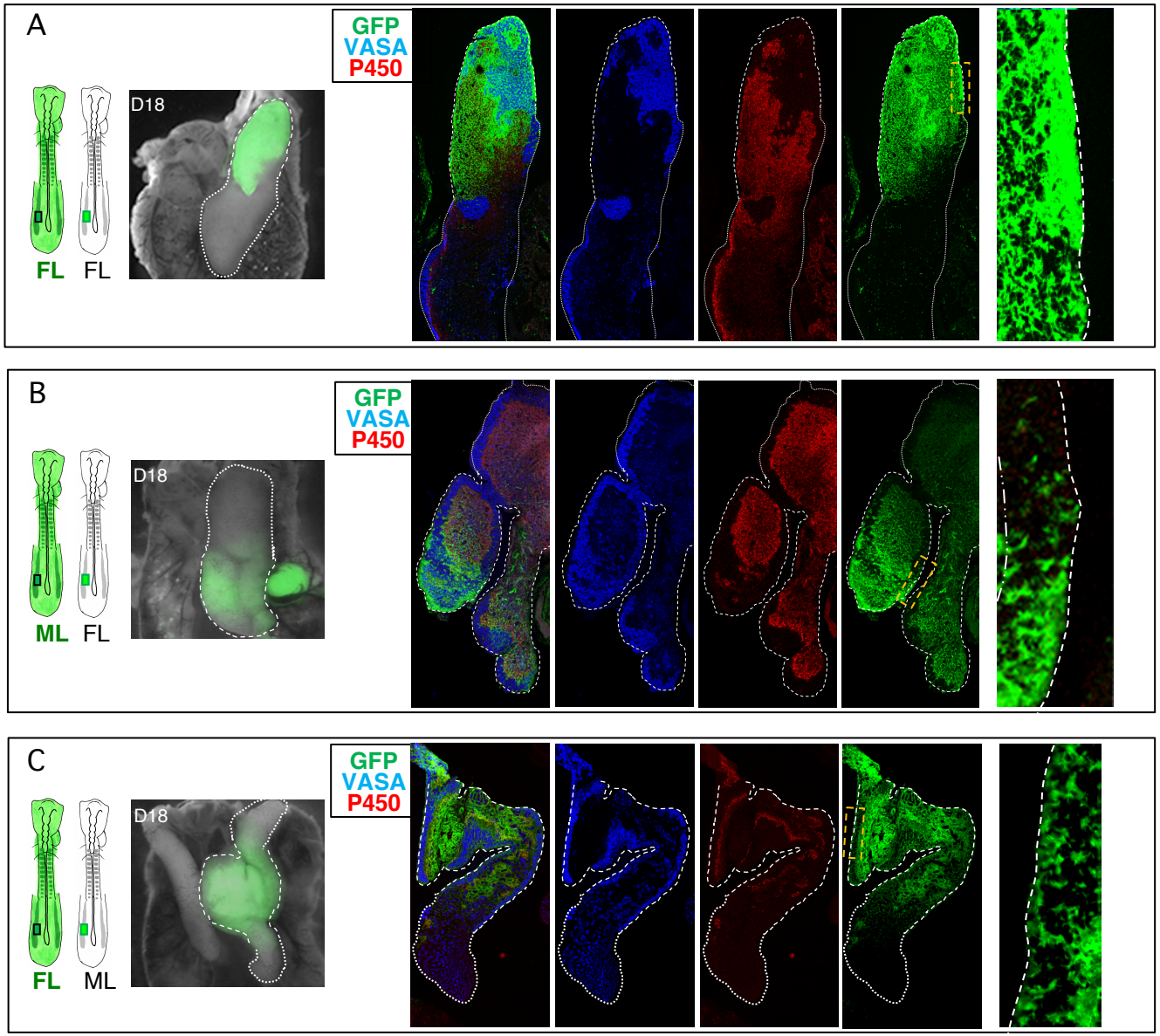
Fig.1

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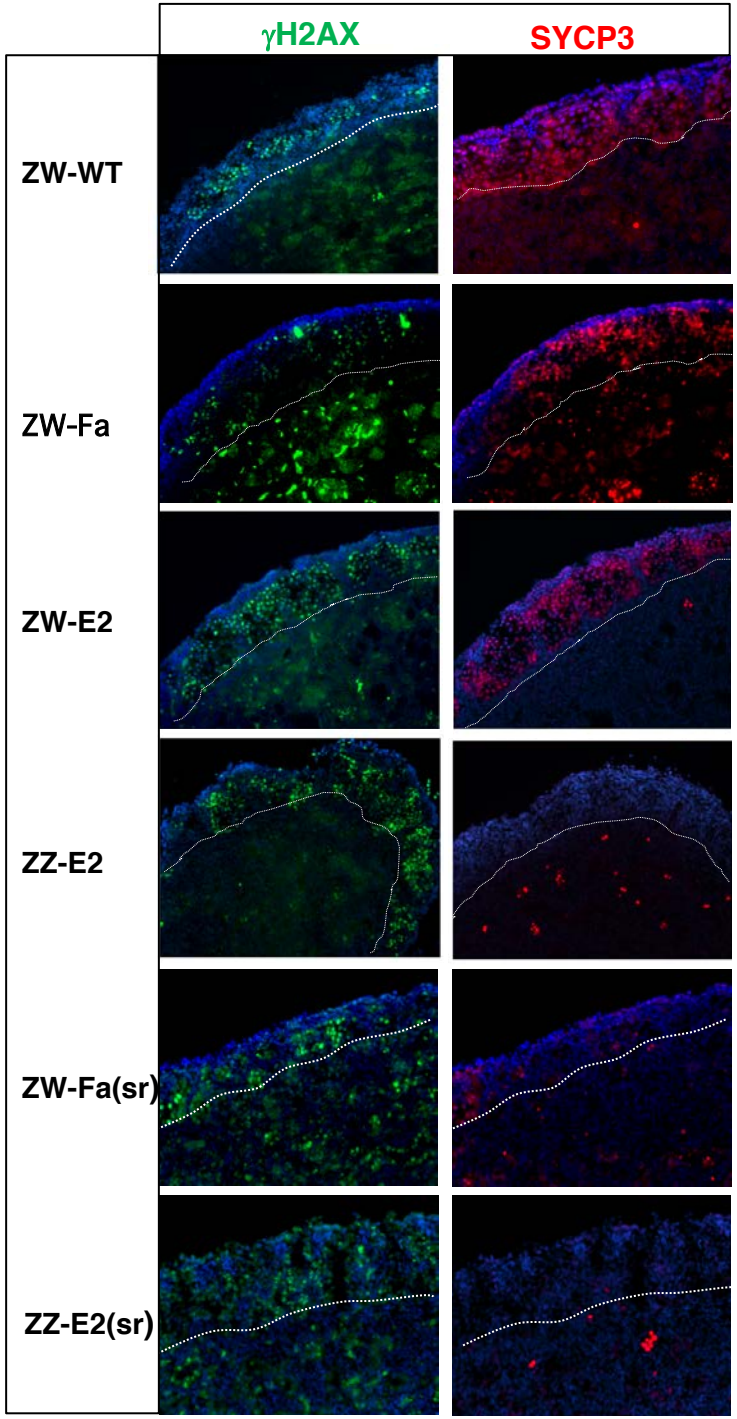

Fig.3

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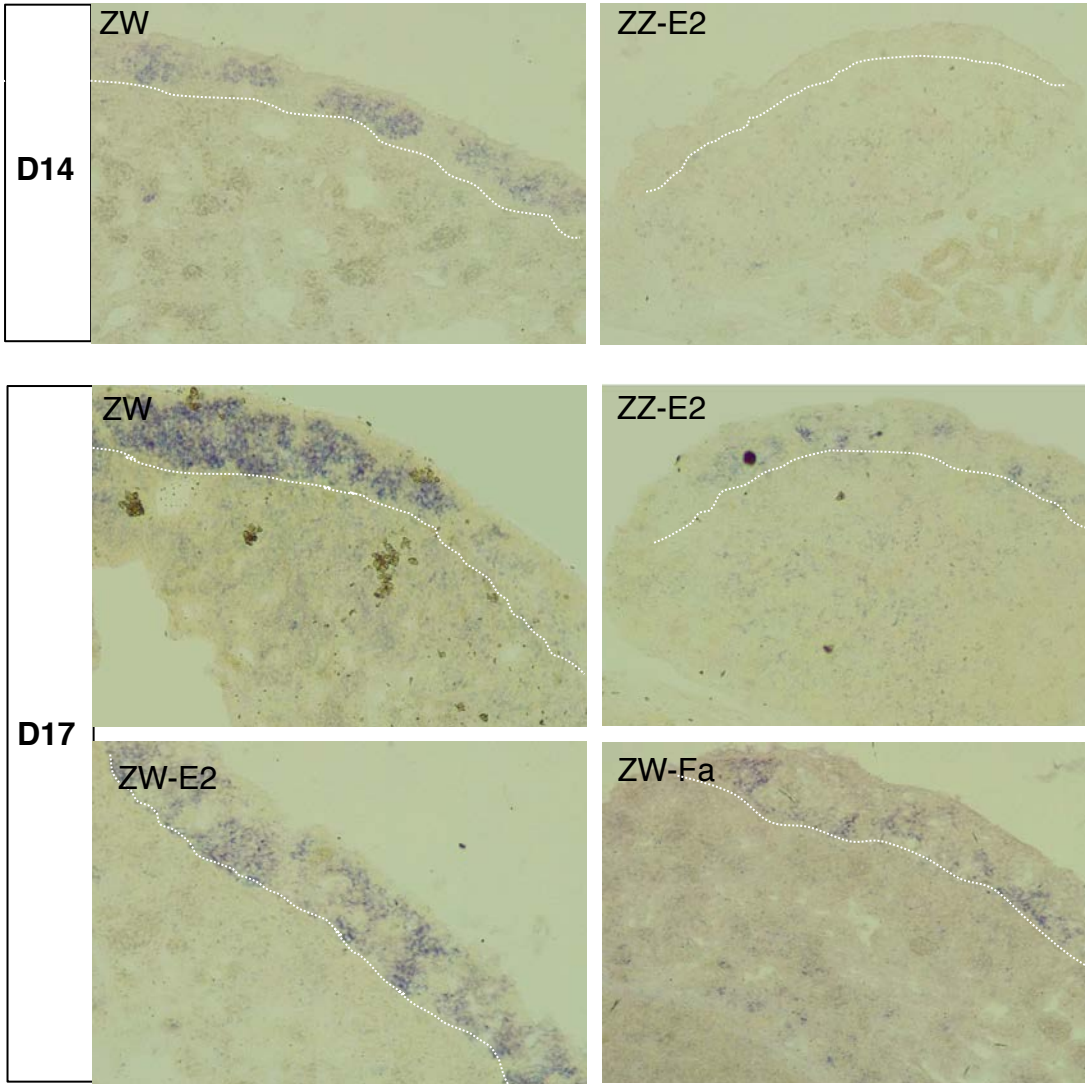

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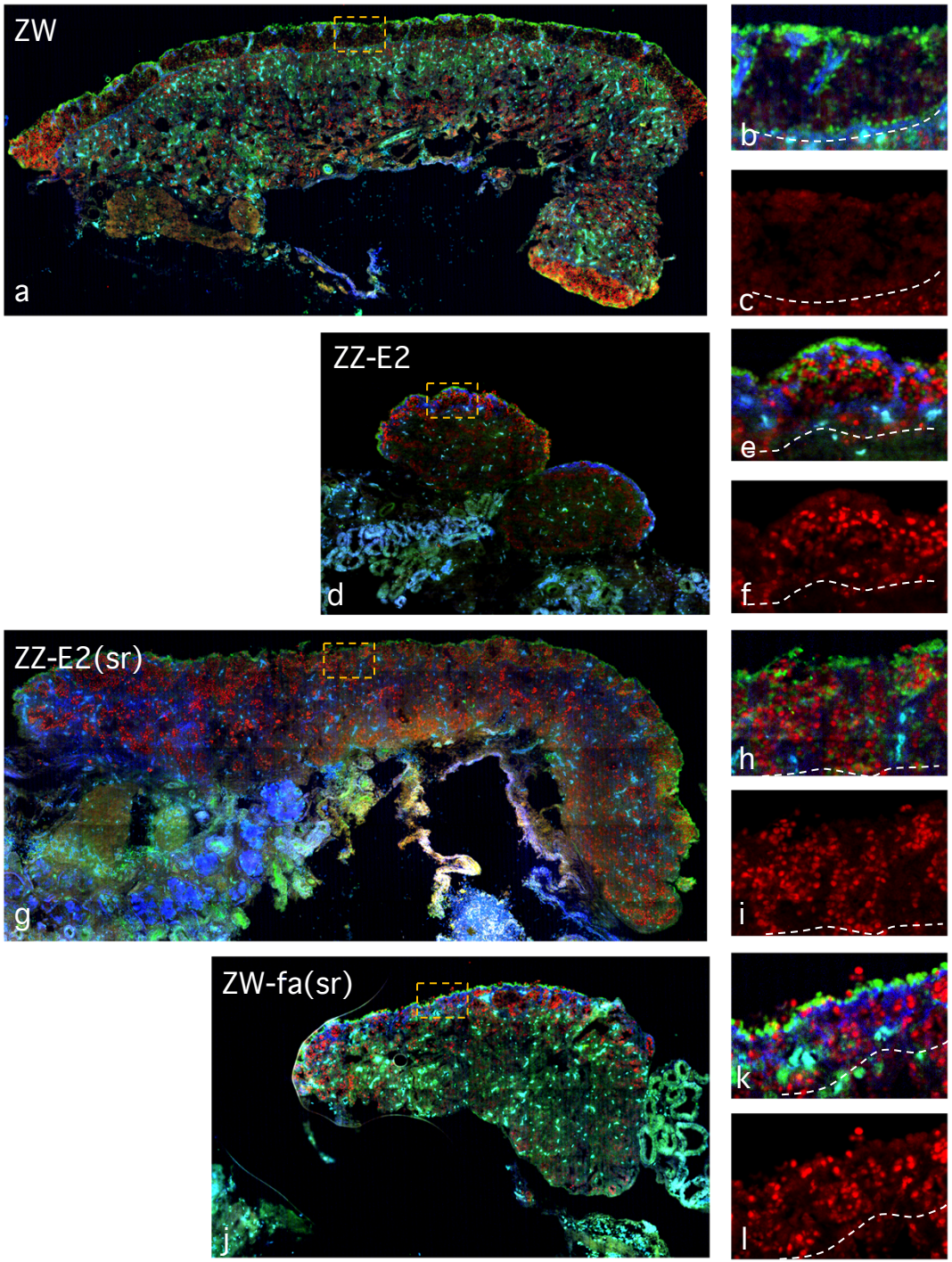

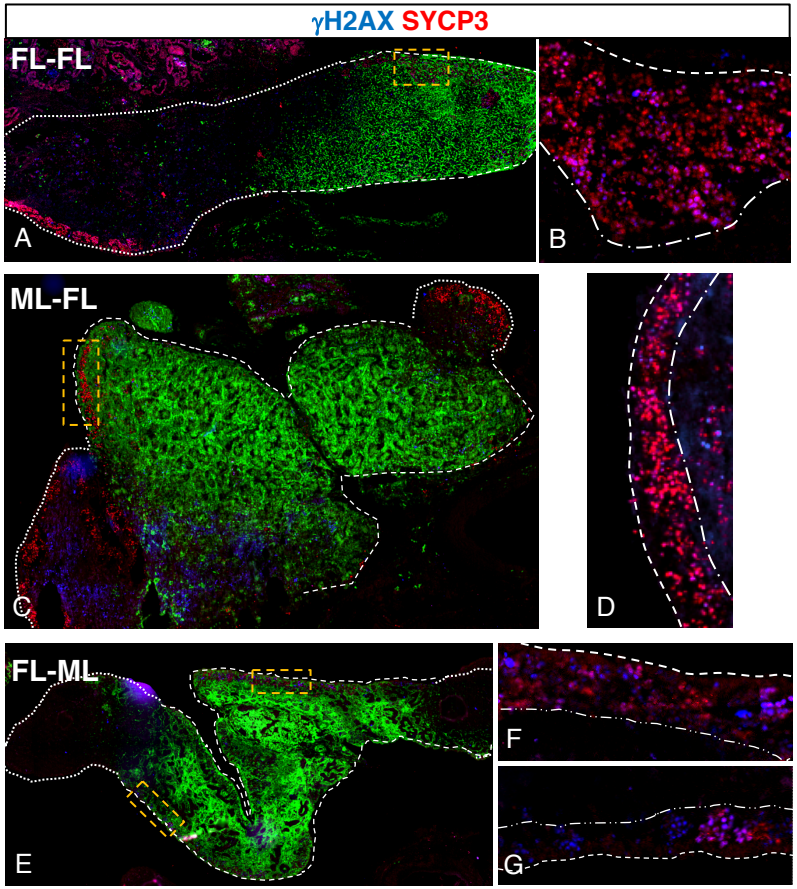

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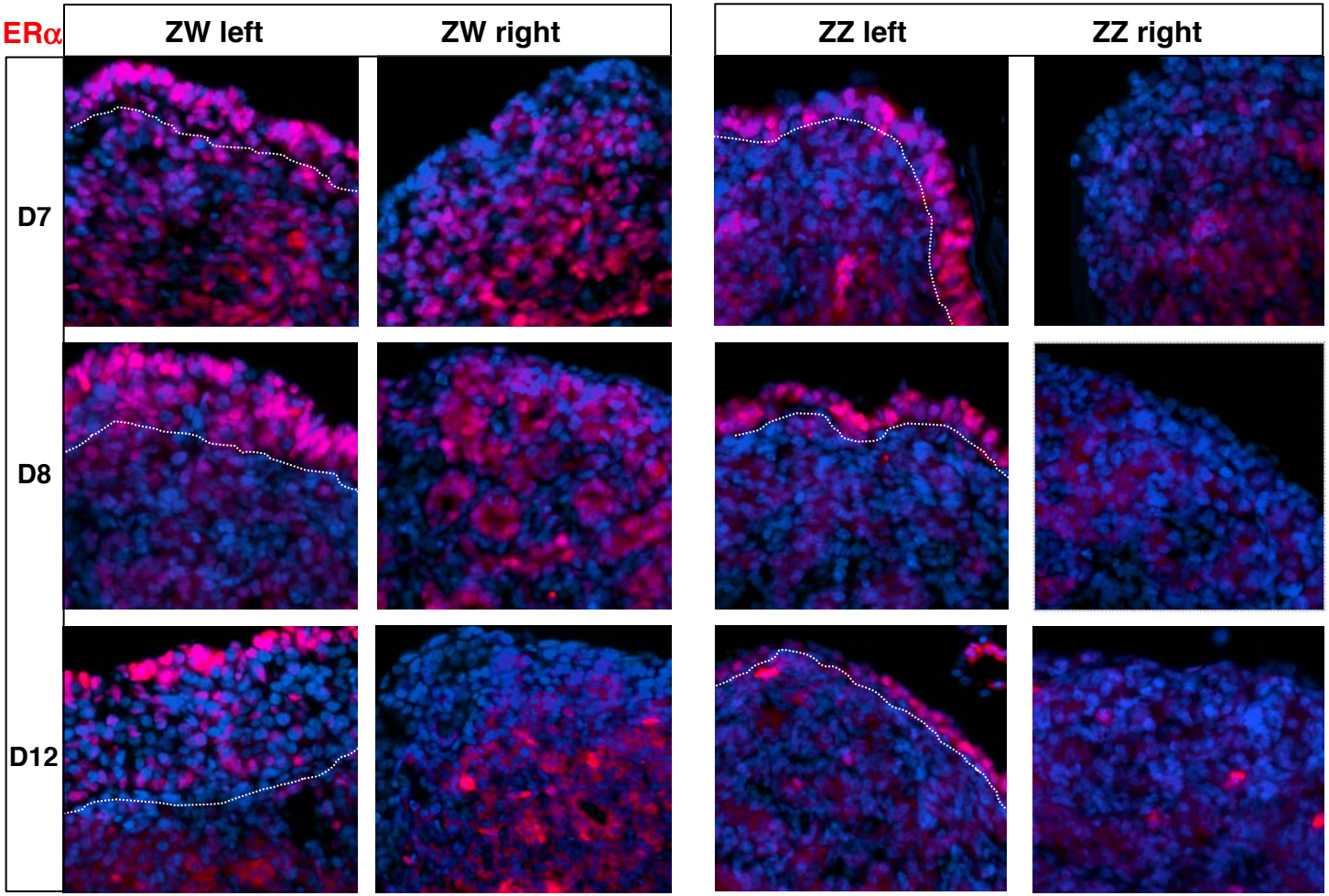

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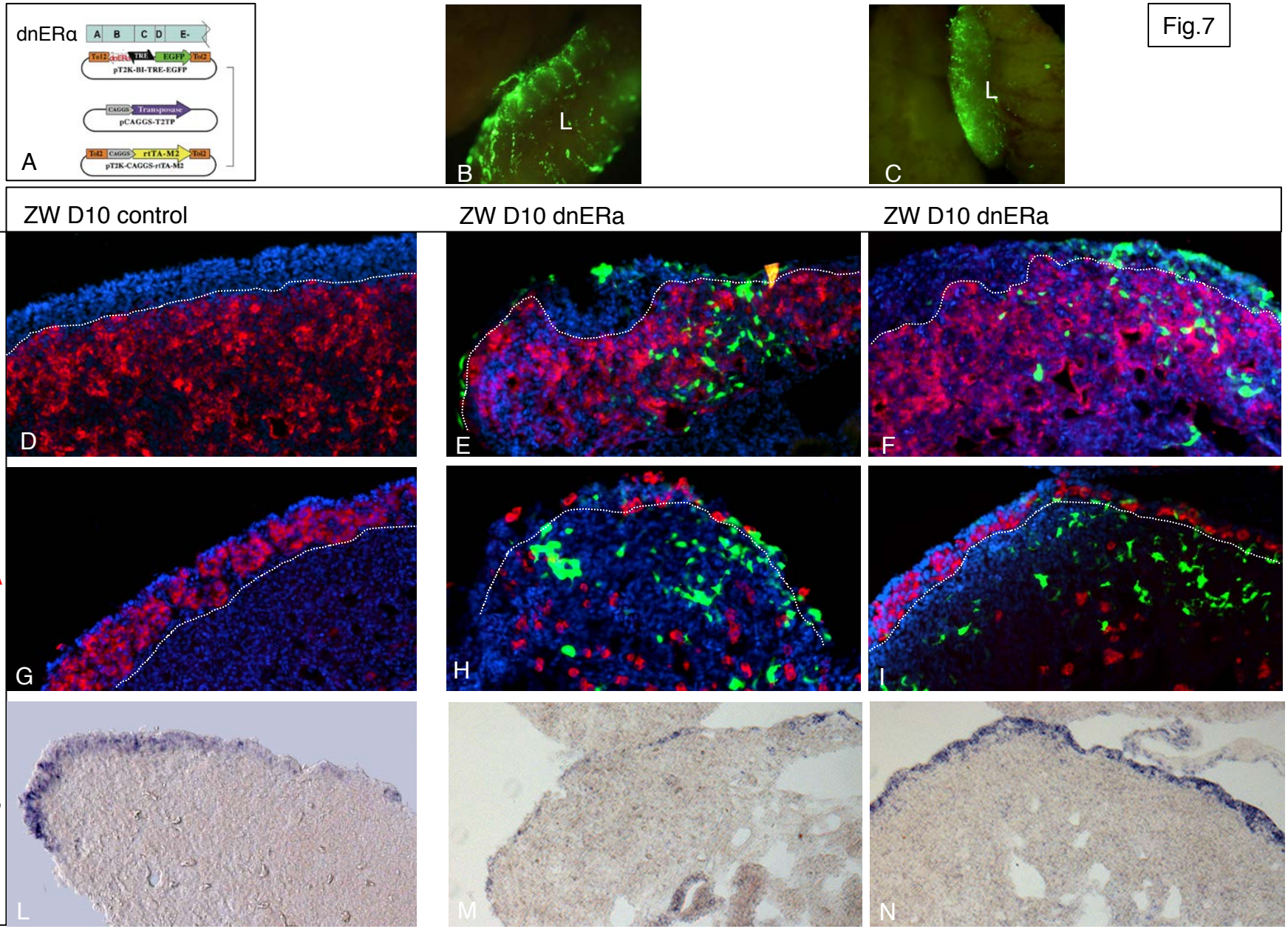
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A
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B
L
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ZW D10 control
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