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Detailed spatiotemporal expression of Prmd1/Blimp1 binding partners during chick embryonic development

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Detailed spatiotemporal expression of Prmd1/Blimp1 binding partners during chick embryonic development.

Degree of Master of Science by Research

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1/26/2015



A Dissertation submitted to the Faculty of Science, University of Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

RESEARCH OUTPUT

Publication:

1. Zwane, T.B.C & Nikitina, N.V. (2015). Spatiotemporal expression analysis of Prdm1 and prdm1 binding partners in early chick embryo. *Gene expression Patterns*, in press.

Conference presentations:

1. Investigation of the role of Blimp1/prdm1 in neural crest development, migration and Epithelial-Mesenchymal Transition (EMT) (2013). Molecular and Biosciences Research Thrust. pp42. University of Witwatersrand [oral presentation]
2. Investigation of the role of Blimp1/prdm1 in neural crest development, migration and Epithelial-Mesenchymal Transition (EMT) (2014). South African Society of Biochemistry and Molecular Biology. pp139. Goudini Spa, Rawsonville. [poster]

DECLARATION

I declare that this Dissertation is my own unaided work. It is being submitted for the Degree of Master of Science at the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



Signature of candidate

Day of 27 March in 2015

ACKNOWLEDGEMENTS

I would first like to thank God for the opportunity I have had to even get this far in my career. Secondly I would like to convey my sincere gratitude to my family, especially my mother who has always believed in me and pushed me to be the best I can be. I also thank my brothers and sisters for continued support even when things were hard. I would also like to thank all my friends for also supporting me and lastly thank my supervisor and everyone who contributed to my research project.

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ABREVIATIONS

AER- Apical ectodermal ridge

AIP- anterior intestinal portal

Blimp1- B-lymphocyte induced maturation protein 1

Bi- blood islands

Cm- cranial mesenchyme

Eb- epiblast

Fg – foregut

GRN – Gene regulatory network

HDAC – Histone deacetylases

HF- head fold

HN- Hensen's node

Hb- hypoblast

LSD- Lysine specific demethylase

Lpm- lateral plate mesoderm

Mhb- midbrain-hindbrain boundary

NPB- neural plate border

NC – neural crest

Nt – neural tube

Nne- non-neural ectoderm

N- Notochord

Np- neural plate

PS- primitive streak

PNC- pre- migratory neural crest

Ppr- pre-placodal region

Ps- primitive streak

Prdm1- positive regulatory domain 1

Op- otic placodes

TLE- Transducin-like enhancer of Split

Zp- zona pellucida

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ABSTRACT

Prdm1/Blimp1 is a transcription factor whose mechanism of action is mainly repression; however it has been identified as an activator in some cases. As a transcriptional repressor, it plays multiple roles during embryonic development, including neural crest specification. Prdm1 acts by repressing large sets of genes via sequence specific recruitment of co-repressors, many of which are epigenetic modifiers. Neural crest is a transient, migrating cell population that gives rise to a number of diverse cell lineages that form important structures in the vertebrate embryo. Examples of these include peripheral nervous system, melanocytes and cranial cartilage. *Prdm1* is expressed during neural crest specification in *Xenopus*, zebrafish and lamprey. The expression of *Prdm1* had not yet been investigated in the neural crest during chick embryonic development. The mechanism of Prdm1 action or the nature of possible binding partners that mediate its effects in the neural crest had not yet been addressed. Prdm1 binding partners are known to play important roles during embryonic development, yet in many cases no spatiotemporal expression analysis during early vertebrate development has been performed. Single and double *in situ* hybridization for *Prdm1* and all the binding partners was performed to determine localization of mRNA during early stages of chick embryonic development. We report the expression patterns of *Prdm1* and seven of its known or putative binding partners (*Hdac1, Hdac2, Tle1, Tle3, G9a, Prmt5* and *Lsd1*) during early stages (HH4-HH10) of chicken embryogenesis. Prdm1 expression was observed in the neural plate border and pre-migratory neural crest during chick development. Six Prdm1 binding partners (except Tle1) are co- expressed with *Prdm1* in the prospective neural plate border at HH4-HH6, and all seven show strong and specific expression in the neural plate border at HH7-HH8, suggesting all of them co-operate with Prdm1 during neural crest development in chick embryos. Future work will focus on protein interaction studies in order to directly demonstrate the association between Prdm1 and the binding partners it co-localizes with.

CHAPTER 1

1. Introduction

1.1. Neural crest (NC)

The neural crest (NC) is a transient embryonic cell population that forms at the apex of the neural tube where it folds in on itself (Basch et al., 2004). Before the neural crest forms, the neural plate is first formed which is followed by the induction of the region known as the neural plate border (NPB) separating the non- neural ectoderm and the neural ectoderm as demonstrated below in Fig.1 and 2. The neural plate border cells form neural folds at the dorsal part of the embryo during the process known as neurulation. These cells delaminate and migrate to different parts of the embryo through a process called the epithelial-mesenchymal transition (EMT). Upon arrival to different sites they then differentiate into a number of derivatives those later form important structures of the embryo (Fig. 1). Examples of these derivatives are neurons, cartilage, melanocytes, glia of the peripheral nervous system (PNS), secretory cells and the craniofacial skeleton. Depending on where the NC cells originate along the anterior-posterior axis they are termed cranial, trunk, vagal or sacral NC. For example cranial NC differentiates to form skeletal tissues that are essential for the formation of the skull (Chambers & McGonnell, 2002). NC is a cell population that is unique to vertebrate embryos and has been studied extensively using fish, lamprey and chick as model organisms (Betancur et al., 2010). To gain a better understanding of the induction signals and molecular pathways underlying the NC formation, a putative neural crest Gene Regulatory Network was proposed (Meulemans & Bronner-Fraser., 2004) (Fig. 3).

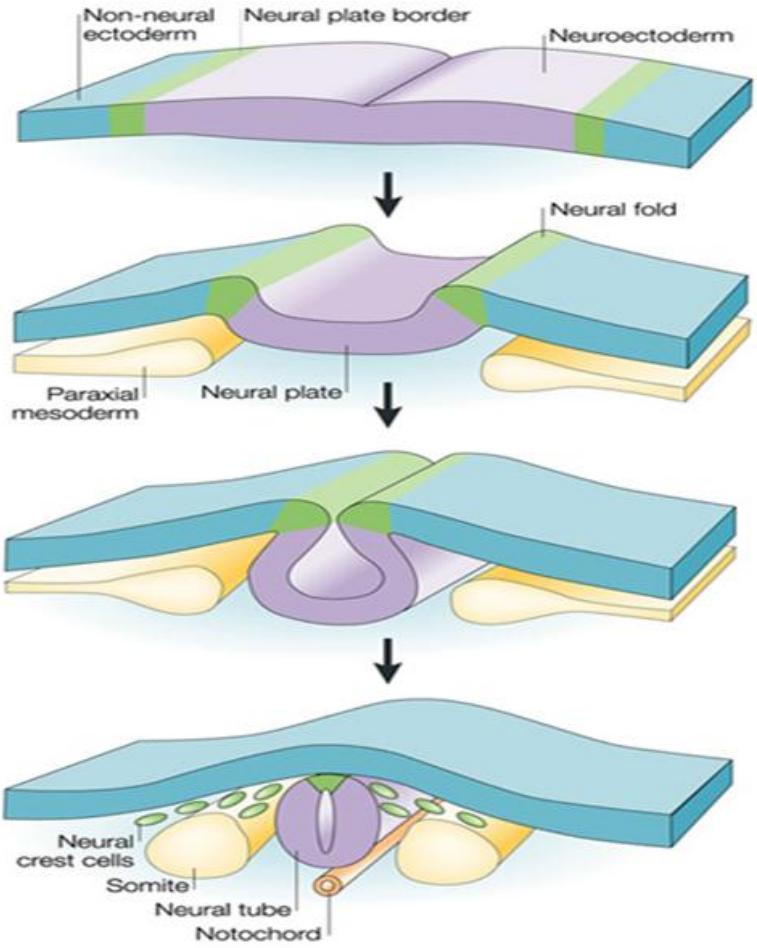


Figure 1: The process of neurulation which leads to the formation of neural crest and the various cell types formed after migration to specific regions in the embryo. Adapted from Knetch, 2002.

There are four sets of genes that are involved in the development of NC and therefore comprise the NC GRN. The first set of genes is the Induction signals which are the early diffusible signals that establish the neural plate border. The second set is the neural plate border (NPB) specifiers which establish the distinct molecular identity of the NPB. The third set comprises neural crest specifiers that are activated in emergent NC cells. The last set of genes is the effector genes which are important in conferring certain properties such as migration and multipotency.

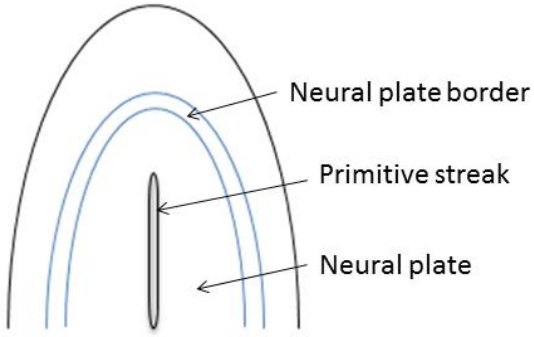


Figure 2: Schematic diagram of a typical stage HH4 embryo during neural plate formation. This image was generated using Microsoft word.

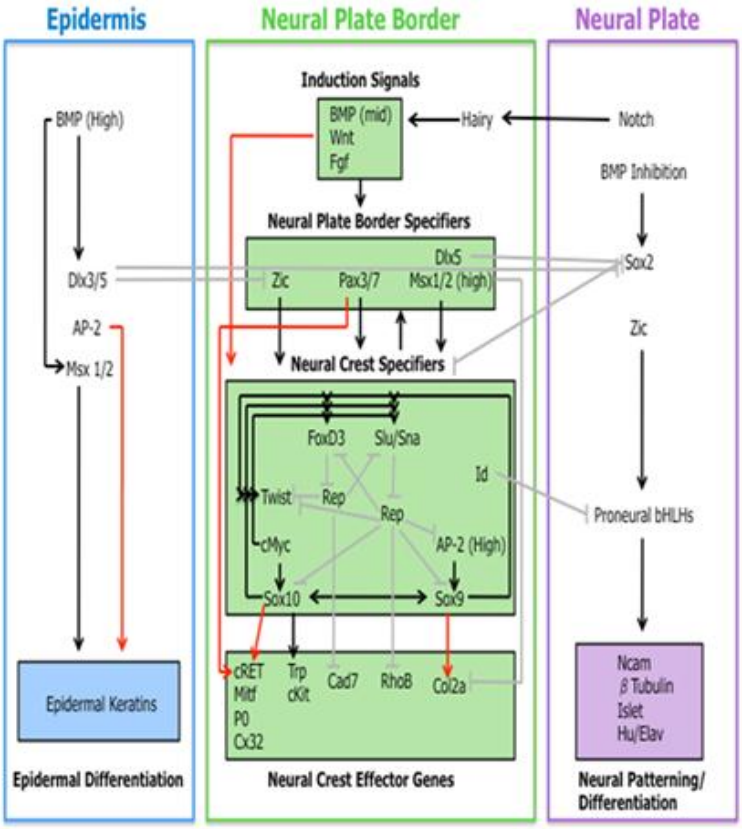


Figure 3: Neural crest Gene Regulatory Network (GRN); showing genes that are involved in the specification of the neural plate border and the neural crest during neurulation. This network includes various transcription factors, induction signals as well as effector gene. Adapted from Meulemans and Bronner-fraser, 2004.

Different induction signals and transcription factors are responsible for the formation of NPB and NC cells. The neural crest gene regulatory network is not yet complete; new transcription factors and epigenetic modifiers involved in the specification of neural crest are still being discovered. A number of genes have been found to be expressed in NC precursors and crucial for NC development i.e. *Snail/Slug*, *FoxD3, Sox10* and *9* etc. These genes play distinct roles as markers of the NC however the relationship between these genes is not yet clear. Genes that are regarded as neural crest specifiers are expressed as early as in the neural folds before complete folding takes place e.g. *Slug*/*Snail*. Our preliminary experiments indicate that *Prdm1* is expressed in the NPB as well as NC of the chick embryo (Fig.5), and that loss of *Prdm1* results in the delay in NC migration (Fig. 6). This preliminary data suggests that Prdm1 might be involved in establishing the neural crest fate and migratory properties in the chick embryo.

NC is not a defined cell population until cells begin to emigrate from the neural folds. Cells within the neural plate border (pre-migratory NC) can also contribute to the neural tube and epidermis (Gammill & Bronner-Fraser, 2003). The first population of cells to delaminate and migrate are those in the cranial (head region) and this happens upon the fusion of the neural folds in birds. Interestingly enough in *Xenopus* and mouse that is not the case, delamination starts while the neural folds are still open and have not yet fused (Theveneau & Mayor, 2012). However, for all animal models, delamination of NC cells in the trunk level happens progressively depending on the organism and formation of certain structures. For example, in chick, the rostral NC cells delaminate after complete closure of the neural tube and caudal NC delaminate a day after completion of neurulation. Delamination at this level correlates with that of somitogenesis where NC cell delamination is before the early differentiation of somites (Sela-donenfeld & Kalcheim, 2000).

Neural crest cells adopt a cooperative behaviour with other neighbouring cells, the local extracellular environment as well as with each other. They migrate through the periphery to their final destinations where they form an array of cell types that differentiate into structures that have been previously mentioned. Some of the molecules that were lost before migration are then restored after NC cells have

stopped migrating e.g. Cadherins. Proper genetic regulation of NC development and migration ensures that appropriate derivatives are formed at the correct time and place. If these cues are not properly regulated, defects in the structures of NC origin are observed. These disorders are collectively known as neurocristopathies and some of the common examples include cleft palate and Hirchsprung's disease. The severity of some of these disorders, which are usually not easy to cure, highlights the importance of studying and understanding the NC cell population.

Gallus gallus (chicken) provides a perfect model organism for this study. The advantages of using chicken are attributed to the following facts: it is oviparous, developing time is shorter and eggs are easily accessible, and it is also evolutionary closely related to humans. Since Prdm1 is not expressed in the neural plate/neural crest in the mouse, this renders the mouse embryos unsuitable for this research.

1.2. Prdm1/Blimp1: Structure and Function

B-lymphocyte induced maturation protein 1 (Blimp1) is a murine homolog of the human positive regulatory domain containing 1 zinc-finger (PRDM1) also known as the PRDI –binding factor 1 (PRDI-BF1). It is a transcriptional repressor that is important for terminal differentiation of B cells to plasma cells (PC) (Calame, 2010). Prdm1 was originally discovered to mediate the repression of the β-interferon (IFN-β) and also to control the expression of *c-myc* (Ren, Chee, Kim, & Maniatis, 1999). PRDM1 has a regulatory role (master regulator) as it ensures the elimination of the immature partially activated B cells while allowing B cells that are fully activated to differentiate to PC (Messika et al., 1998). In T cells Prdm1 is expressed in memory and effector cells and it is usually induced upon activation (John & Garrett-Sinha, 2009). A high level of expression has also been reported in a subset of $CD4^+$ and $CD8^+$ T cells as well as in natural killer cells and macrophages (Hohenauer & Moore, 2012). Though it is known as a repressor, it has been reported as an activator in some cases which suggests a bidirectional transcription role. Prdm1 in combination with interferon regulator factor 4 (IrF4) can promote activation in regulator T- cell. The mechanism of activation is through the up regulation of activating H3K4 methylation after H3K27 has been removed in the il10 by Irf4 (Hohenauer & Moore, 2012).

Prdm1 not only plays a role in the immune cell development and function but has been implicated in stabilizing and maintaining the identity of photoreceptor cells (Muncan et al., 2011). This is achieved by binding to the promoters and repressing alternative cell fates. The same mechanism of program switch was observed in the repression of adult enterocytes while promoting the metabolism of neonatal enterocytes (Harper et al., 2011). Prdm1 also plays a role in the development and differentiation of many tissues in different organism e.g. mice. Prdm1 is expressed in a number of tissues during embryonic development (Table 1) and lack of Prdm1 in mice resulted in death at embryonic day 10.5. Mutant embryos also exhibited the characteristics of complete loss of Primordial Germ Cells (PGCs), and other defects in placenta and in blood vessels (John & Garrett-Sinha, 2009). Other loss-of-function phenotypes included loss of anterior structures (head) in lamprey, *Xenopus* and zebrafish. Additionally in zebrafish, branchial arch defects were observed. This may have been due to the loss of neural crest cells. Additionally , zebrafish Prdm1 mutants exhibit loss of Rhohon-Beard neurons and slow-twitch muscle cells (Hernandez-Lagunas et al., 2005). Prdm1 is required for specification, development and migration of particular cell types in the developing embryo. Its expression pattern is diverse and across different species during different developmental events. Some of these are documented in the table below (Hohenauer & Moore, 2012).

Table 1: Prdm1 expression patterns across different species during embryonic development.

Organism	Expression pattern	References
Mouse	Anterior endoderm, prechordal plate, head mesoderm, Primordial germ cells	(Chang, Angelin-duclos, & Calame, 2000; Robertson et al., 2007)
Zebrafish	Somites, retina, Rhohon-beard sensory	Sun et al., 2008; (Roy

	neurons, prechordal plate	& Ng, 2004)
Xenopus	Limb buds, pharynx, sensory neurons, neural crest	De souza et al., 1999; Rossi et al., 2008
Sea urchin	endomesoderm	Livi and Davidson, 2006
Starfish	endomesoderm	Hinman et al., 2003
Lamprey	Neural crest (premigratory), somites, eye	Nikitina et al., 2011

Owing to the diverse and important roles it plays during embryonic development as well as in adult tissue, it is important to understand its mechanism of action. Prdm1 mechanism of repression is not clearly understood at the moment however it appears to associate with a number of co-repressors in a tissue specific manner. Its structure enables it to bind to promoter regions and repress a large subset of genes by recruiting other chromatin modifying enzymes. As a transcriptional repressor Prdm1 has to form a number of complexes through its binding domains in order to perform its function.

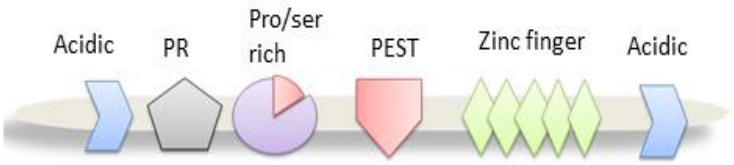


Figure 4: Prdm1 structure with different binding domains. Acidic domains on the N terminal and the C terminal (blue), PR domain (dark grey), Proline/serine rich domain (purple), PEST domain overlapping with Proline rich as well (red) and the Zinc fingers (light green). This figure was generated using Microsoft word.

PRDM1 is a 98kDa protein encoded by the *Prdm1* gene found on the long arm of chromosome 6 (6q21). Prdm1 is part of the PRDM family of proteins which are characterized by the N-terminal PR domain as well as the zinc finger motif with the exception of a few protein members (Hohenauer & Moore, 2012). Prdm1 has other domains which are discussed in detail below (Fig. 4 and 5).

Different protein isoforms of Prdm1 can be generated by the *Prdm1* gene and these are due to alternative splicing. Alternate splicing of exon 7 in mice leads to a protein

that lacks the portions of zinc fingers (1&3 and no 2nd zinc finger) (John & Garrett-Sinha, 2009). Though this isoform is not able to bind to DNA, it was shown to be expressed in immature B cells where it affected apoptosis and also inhibited the activity of full length Prdm1. It was presumed to inhibit the activity of full length Prdm1 by forming non-functional heterodimers (John & Garrett-Sinha, 2009).

1.3. Prdm1's role in the neural crest (NC)

As a transcriptional regulator it represses a large subset of genes thus instructing specific cell fate and repressing other alternative cell fates during vertebrate development (Brzezinski et al., 2013). Some of the genes that are known to be targets of Prdm1 repression during plasma differentiation are MYC, MHC2TA and PAX5 (Mora-López et al., 2008). Prdm1 is involved in the specification of neural crest in zebrafish but is not expressed in mouse NC. The expression of *Prdm1* in mouse was only observed in E10.5 on branchial arches and loss of function resulted in the loss of branchial arch posterior to the first arch (Birkholz et al., 2010). *Prdm1* does not seem to be expressed in the mouse neural plate (NP) and does not appear to be required either for the neural crest (NC) or muscle cell specification (Chang et al., 2002; Vincent et al., 2005; Robertson et al., 2007)

Prdm1 plays an important function in the specification and development of neural crest in a number of organisms. In zebrafish the Prdm1 homolog *u- boot* (*ubo*) is involved in the specification of NC and sensory neurons specifically the Rohon-Beard (RB) sensory neurons (Roy & Ng, 2004). The expression of *ubo* was observed as early as the neural plate border; however there was no expression in the developing RB and NC. In both the hypomorphic Prdm1 mutant,narrowminded, and the null mutant, U-boot, there is a decrease in the number of neural crest cells, and all the neural crest derivatives are much smaller than in the wild-type (Artinger et al., 1999; Roy and Ng, 2004; Hernandez-Lagunas et al., 2005). The incomplete loss of NC cells in these mutants can be attributed to the fact that there are four Prdm1 paralogs in zebrafish, so that at least partial functional compensation might be occurring. It is also required for proper development of the slow-twitch muscle and fin bud outgrowth however is not important for development of germ cells in

zebrafish (Hammond et al., 2009). A recent report from Artinger lab (Powell et al., 2013) demonstrated that Prdm1 can serve as both a transcriptional activator and transcriptional repressor during zebrafish neural crest development, and that it is able to directly bind and activate the enhancers of two essential neural crest specific transcription factors, foxd3 and tfap2.

Expression patterns as well as the loss of function studies prove that Prdm1 plays various roles in the developing embryo; some of these roles are conserved and are similar in different organisms. Even more so there seemed to be a conserved role in NC specification and development and this has been observed in *Xenopus*, zebrafish and in the most basal extant vertebrate lamprey. *Prdm1* is expressed in the pre-migratory NC population in lamprey (Nikitina et al., 2008).

Published data indicated that *Prdm1* was expressed in the chick embryo at stages HH14-17 in these structures: developing eyes, branchial arches and otic placodes (Ha & Riddle, 2003). It was also expressed in the limb bud of a stage HH18 embryo; however no expression was reported during early stages (HH4-HH10). Therefore is not known to date if Prdm1 plays a role in the specification and development of NC. Prelimenary data from our lab indicates that *Prdm1* is expressed in the neural plate border, where NC specification occurs (Fig. 6). *Prdm1* expression was also observed in the pre-placodal region (ppr), head ectoderm and the in the germinal crescent cells. Its expression is turned off in the neural crest around stage HH9 as seen in Fig.6.

1.5. Prdm1 binding partners

1.5.1. Interactions with Prdm1

As a master regulator, Prdm1 regulates transcription by interacting with a number of epigenetic modifiers that help re-organize chromatin at target sites. Most of these epigenetic modifiers/binding partners are co-repressors e.g. histone deacetylases, methyl transferases and the Groucho family of proteins. Some of these interactions were identified in B-cells e.g. G9a, LSD1, TLEs and HDAC2 (Bikoff et al., 2009). Prmt5 which is an arginine methyltransferase plays a role in the germ cell lineage.

These interactions seem to be tissue specific and therefore it is possible that not all these binding partners associate with Prdm1 in all tissues where Prdm1 is expressed. To elucidate the function of Prdm1 in other tissues where is has not yet been documented, these interactions first have to be explored.

1.5.2. Nature of interaction and binding domains

Prdm1 has multiple binding domains that it uses to associate with a different number of co-repressors. These domains are shared across the PRDM family of proteins.

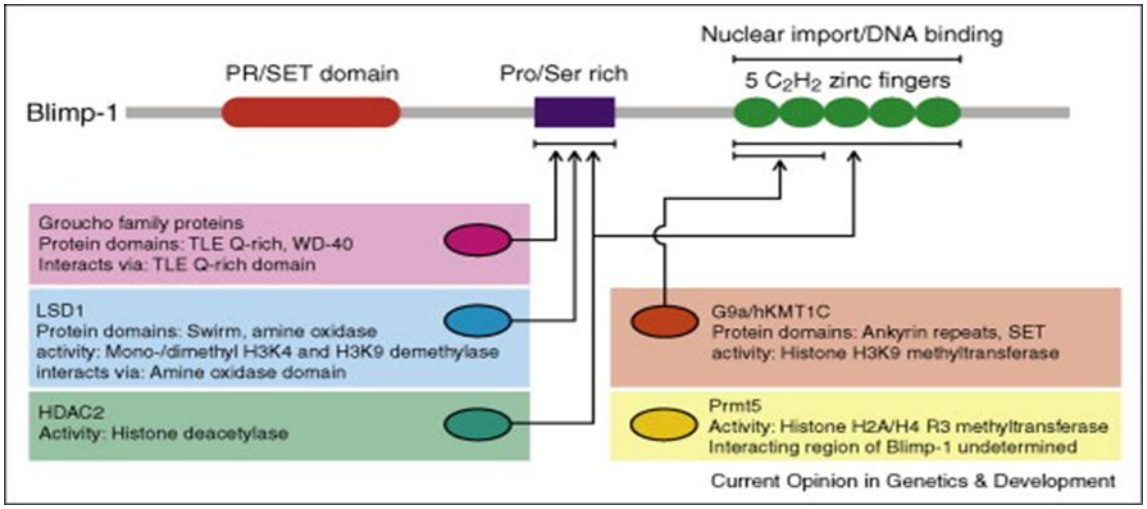


Figure 5: Binding domains and the proposed interactions with some binding partners. Different chromatin modifying enzymes bind to different regions; however some may use the same region, adapted from Bikoff, 2009.

PR/Set domain is found on the N-terminus and has intrinsic/ endogenous methyltransferase activity found in most PRDM family members however, Prdm1 lacks this activity (Hohenauer & Moore, 2012). The second domain closer to the PR/ SET domain is the proline/serine rich domain which is important for binding to other co-repressors that are recruited to target site. This domain is mainly involved in the binding of the Groucho family proteins, HDAC1/2 and LSD1. The main characteristic domain for this protein family is the 5 zinc fingers that are used in nuclear import and DNA binding. The first 2 zinc fingers (towards the N terminus)

are also used to associate with G9a; these have been proposed to also require the proline/serine rich domain (Su et al, 2009) (Fig. 5).

1.5.3. Functions of Prdm1 binding partners in embryo development

Binding partners are mostly histone modifying enzymes that are recruited by Prdm1 to the target promoters. However these co-repressor elements also work independently of Blimp1 and have various other functions in other tissues. In some cases the embryonic expression and function of these co-repressors has not yet been elucidated which makes them of particular importance for our studies.

a) Groucho family/ Transducin-like enhancer of split (TLEs)

Transducin-Like Enhancer of split (Tle) proteins (also known as Groucho-related proteins) are a family of proteins which is mainly involved in the repression of target genes by associating with DNA binding proteins i.e. Prdm1 (Chen & Courey, 2000). Gro/TLE family consists of five members that have been identified and documented so far, and these include TLE 1-4 and amino enhancer of splits (AES). The involvement of the Gro/Tle family has been observed in a number of developmental processes, including lateral inhibition, segmentation, sex determination, dorsal/ventral pattern formation, terminal pattern formation and eye development. The expression has been observed both during embryonic development and in the adult. Gro/TLEs are characterized by the WD40 repeat on the carboxyl terminus which are proposed to be used for protein-protein interactions (Fisher & Caudy, 1998).

The general mechanism of function for this family of proteins is active repression of both activated and basal transcription. However Tles do not have DNA binding domains and therefore do not bind DNA directly, but require targeting to the template DNA by partner proteins (Chen & Courey, 2000). This suggests that they do not compete with transcriptional activators or quench their activation domains. Groucho proteins are recruited by an array of DNA binding proteins (transcription factors) i.e. c-myc, Pax, Six etc. (Jennings & Ish-Horowicz, 2008). Mammalian TLEs can use a number of different DNA-binding proteins as co-repressors,

including Hairy-related transcription factors, Aml1 and Prdm1. Tles serve as regulators of several signalling systems including the Notch, Wnt and Bmp/TGF-beta. Interestingly, TLEs appear to be able to interact with Hdac1 and recruit it to specific DNA sequences, resulting in histone deacetylation and chromatin silencing (Turki-Judeh & Courey, 2012). In the context of human B cell development, PRDM1 was demonstrated to directly bind TLE1 and TLE2 (Ren et al., 1999), however it is possible that other TLEs are able to associate with Prdm1 as well.

Expression patterns of all five *Tles*/ *Gro* genes during embryo development were detected mostly in the neural tube, and the cross sections show that the expression is in the ventricular zone where the proliferating neural progenitors are found (Van Hateren, Belsham, Randall, & Borycki, 2005). These expression patterns were examined by whole mount *in situ* hybridization of quail embryos, and all of the *Tles* were expressed from an early stage 3 to stage 24. Based on these expression patterns it was hypothesised that *Tle* genes are not only involved in the central and peripheral neurogenesis but are also important in segmentation and epithelial differentiation (Grbavec et al., 1998).

The focus of this study was on Tle1, Tle3 and Tle4 in the development of chick embryo. The main focus of our study was based on co-expression of the above mentioned *Tles* with *Prdm1*. Previously published data shows ubiquitous expression in the earlier embryonic stages (Van Hateren et al., 2005). This could be due to probe cross-hybridization or poor performance of the *in situ* hybridization protocol. We decided to verify *in situ* hybridization for TLEs using early stage chick embryos (HH4-HH10).

b) Protein arginine methyltransferase -Prmt5

Prmts are arginine methyl transferase enzymes that are important in chromatin modification leading to controlled gene expression, proliferation and development of the organism. Chromatin modification is achieved by post-translation of histones on their N terminal tails (Karkhanis et al., 2011). Post translational modifications usually involve methylation and acetylation of histone tails and non-histone proteins on certain residues. However arginine methyl transferases add methyl groups on

arginine residues instead of lysine. Prmt5 is a type II methyltransferase and is involved in cellular differentiation, germ cell specification, Golgi apparatus assembly and ribosome biogenesis (Karkhanis et al., 2011). Prmt 5 associates with many complexes such as the SWi/SNF chromatin remodelers, and silences tumour suppressor genes and other genes involved in the regulation of the cell cycle.

Prmt5 has a number of binding partners apart from the complexes it associates with. In NURD/ MBD2 complexes Prmt5 associates with MEP50. A temporal association of Prdm1 with Prmt5 was reported in mouse germ cell development; however the protein domain mediating this interaction is still not determined (Ohinata et al., 2005). Proper development of primordial germ cells (PGC) is important to prevent testicular germ cell tumours which could have a fatal outcome in males (Eckert et al., 2008). The suppression of somatic differentiation program in PGCs which could account for seminomas requires the PRDM1/ PRMT5 complex where there is a down regulation of Prdm1 (Eckert et al., 2008). The down regulation of Prdm1 is achieved by translocation of the PRDM1/PRMT5 complex in the cytoplasm upon PGC differentiation. Loss of Prdm1 by deletion results in loss of PGCs and there is an insufficient repression of markers that are necessary for somatic differentiation in Prdm1 deficient PGCs (Eckert et al., 2008). Therefore PRMT5/PRDM1 complex plays an important role in the development of human germ cell; furthermore this complex is expressed in the seminomas but down regulated in non-seminomatous germ cell tumours. Prmt5 is also found to be elevated in a variety of transformed cells e.g. B- cells (Karkhanis et al., 2011). In complex with PRDM4, PRMT5 maintains stem cell like properties of the neural stem cells (Chittka, Nitarska, Grazini, & Richardson, 2012). In combination with Oct3/4 and Klf4, Prmt5 is able to re-programme mouse embryonic fibroblasts into embryonic stem cell-like state (Nagamatsu et al, 2011). In addition to its role in maintaining pluripotency/ undifferentiated state, Prmt5 appear to promote differentiation of mouse glial cells via epigenetic silencing of Id2 and Id4 (Huang et al, 2011), and induces fetal globin gene silencing in human adult erythroid precursor cells (Rank et al., 2010). A recent study also showed that Prmt5 is important in RNA splicing which enables MVH negative cells to become MVH positive gonadal stage germline cells (Li et al.,

2014). Surprisingly, no detailed *Prtm5* expression pattern in mouse or chick embryos has been reported yet.

c) Lysine specific demethylase (LSD1)

Lysine-specific demethylase 1 (Lsd1 or Kdm1) is a histone demethylase that removes methyl groups from mono- or dimethylated lysine 4 or lysine 9 of histone H3 (Su et al., 2009). LSD1 represses genes by demethylating the mono- or dimethyl groups specifically. It interacts with a number of co-repressors e.g. neural restrictive silencing factor (CoRest) and HDAC 1and 2. It has also been observed to interact with non-histone proteins like p53, where demethylation is at residue K370 which results in reduced interaction with a co-activator p53 binding protein 1(Jing Huang et al., 2007). Su et al reported that LSD1 is required in Prdm1 mediated gene repression and this repression is via the proline/serine rich domain during plasma cell differentiation. This Prdm1 domain was observed to interact with HDAC1/2 and LSD1 in a multi-protein complex (Su et al., 2009).

Lsd1 demethylates histone H3 on lysines 4 and 9 (H3K4/H3K9) during mouse embryogenesis (Wang et al., 2009). It also forms the component of NurD complex, which is essential for the differentiation of embryonic stem cells (ESC) (Whyte et al., 2012). Mixed lineage leukemia (MLL) gene is repeatedly rearranged in leukaemia. Lsd1 demethylates H3K4 methylation catalysed by the MLL gene which suggest LSD1's involvement in cancer development (Hu et al., 2009). Hematopoietic development is an important process where LSD1-mediated epigenetic modification also plays a role. This was confirmed by the down regulation of LSD1 by siRNA in hematopoietic lineages (Hu et al., 2009).

Relatively little is known about the role of this epigenetic modifier in vertebrate development. As a part of LSD1-CoREST complex, Lsd1 represses neuron-specific genes in non-neuronal tissues and neuronal precursors (Ballas et al., 2001). Lsd1-null homozygous mice die by E7.5, however, conditional deletion of Lsd1 in the mouse pituitary gland results in the failure of late cell-lineage determination and terminal differentiation. Interestingly, it appears that Lsd1 can act either as a co-repressor or a co-activator, depending on its binding partners (Wang et al., 2007). To

the best of our knowledge, there are no published reports of *Lsd1* mRNA spatial expression in any vertebrate embryo.

d) Histone deacetylases (HDAC1 and 2)

Histone deacetylases are chromatin modifying enzymes that produce repressed gene state. Histone deacetylases act by removing acetyl groups from histones thus producing repressed gene states. Eighteen deacetylases have been identified in mammals and they are divided into 4 classes based on sequence similarity (Brunmeir et al., 2009). Class I has 4 members namely HDAC1, HDAC2, HDAC 5 and HDAC8. HDAC1 and 2 have a high sequence similarity (82%) and in most organisms are found together in certain complexes mentioned below with a few exceptions.

This high similarity may suggest overlapping function however some knock out studies suggest that the function is distinct in certain organisms.

There are several classes of histone deacetylases, the main focus regarding this project was on Class 1 HDACs in particular HDAC1 and HDAC 2. HDAC1 and HDAC2 evolved in a recent gene duplication event which would explain the 80% similarities in these genes. They are mostly found together within the same transcriptional repressor complex indicating a high degree of redundant function (Brunmeir et al., 2009; Murko et al., 2011). HDAC1 plays a role in the embryonic stem cells and it was demonstrated that it was the major HDAC in the critical pre-implantation development in mouse (Pillai, Coverdale, Dubey, & Martin, 2004). During embryo development HDAC1 is expressed at sites of anterior and posterior neural tube closure of stage 12 chicken embryo (Murko et al., 2011). Both HDAC1 and HDAC2 were found to be expressed in the developing brain and a higher level of expression was seen in the forebrain regions and decline in the hindbrain from stage 12. Therefore the distinctive expression of both HDACs in the central nervous system (CNS) is indicative of the cell-autonomous role of HDAC1 and 2 (Murko et al., 2011).

HDAC1 and HDAC2 are often found in the same repressive complexes, e.g. NuRD, CoREST, NODE and SHIP (Brunmeir et al., 2009); however, Hdac1 and Hdac2-null

mice exhibit different phenotypes i.e. disturbed allantois formation and embryos show retarded growth. This suggests limited functional redundancy for these two acetylases during mouse embryogenesis (Lagger et al., 2002). *Hdac1* and *Hdac2* expression in chick embryo was examined by quantitative RT-PCR at HH2-HH17, and by *in situ* hybridization from HH12-HH25; however, no spatiotemporal expression analysis of *Hdac1/2* was reported during early stages of chick embryogenesis (Murko et al., 2010).

e) Lysine specific methyltransferase G9a

G9a (or EHMT2/Kmt1c) is a methyltransferase that is responsible for dimethylation of histone H3 at lysine K9, and for trimethylation of H3K27 to H3K27Me3, resulting in heterochromatin formation. G9a is important for the euchromatic histone H3K9 methylation that is essential for early embryogenesis, the propagation of imprints and control of DNA methylation. G9a has been reported to function in genomic imprinting and associate with partners other than Prdm1 e.g. C/EBPβ to repress transcription via methylation.

Repression by G9a via C/EBPβ was observed when Lys-39 was methylated but it could not be deduced whether G9a acts only to create a new binding site for the repressive protein complex (G9a/C/EBPβ) or it enhances the interaction with C/EBPβ by reading the methylated Lys-39 and thus exerting repression (Pless et al., 2008). It also forms complexes with the GLP through the SET domain interaction, which exists as G9a/GLP heteromer mostly (Shinkai & Tachibana, 2011). In mouse embryos, G9a expression is detected by RT-PCR or immunocytochemistry in many tissues including fetal liver, bone marrow, and developing skeletal muscles, though no comprehensive study of spatiotemporal expression has been published to date (Mei et al., 2012). Loss of G9a in mice results in embryonic lethality by E9.5–E12.5. G9a knockout embryos exhibit higher than normal levels of apoptosis and developmental arrest, so that the mutants at E9.5 morphologically resemble wild type embryos at E8.0–E8.5 (Tachibana et al., 2002). In zebrafish embryos, strong G9a expression is seen in the brain, eye, anterior somites and intestine. Reduction of G9a levels by 37% using a splice blocking morpholino results in a drastic reduction in brain size, abnormal retina development and lethality by 80dpf (Rai et al., 2010).

There is no expression pattern reported for G9a to date in chick embryo development.

1.6. Preliminary experiments/data

Preliminary data from our laboratory demonstrated that *Prdm1* is expressed in the tip of the neural folds, an area that includes neural crest progenitors of a chick. This gene is turned off upon migration of NC which may suggest a role in the EMT mechanism and early steps of neural crest specification (Fig. 6). Loss-of-function experiments where Blimp-1morpholino (MO) was used to prevent Prdm-1 translation demonstrated a delay in neural crest migration (Fig.7). In this experiment, Prdm1 morpholino oligonucleotides (MO) were first injected in one side of the E4 embryonic ectoderm, while the other side was not injected. This was to monitor the differences that occur on the injected side. Again another embryo was injected with control MO while the other side was not. After the injection, voltage was applied to allow MO to be taken up by the cells. Embryos were allowed to develop to HH10/11, and NC cells were detected by immunostaining for HNK and or Pax7.

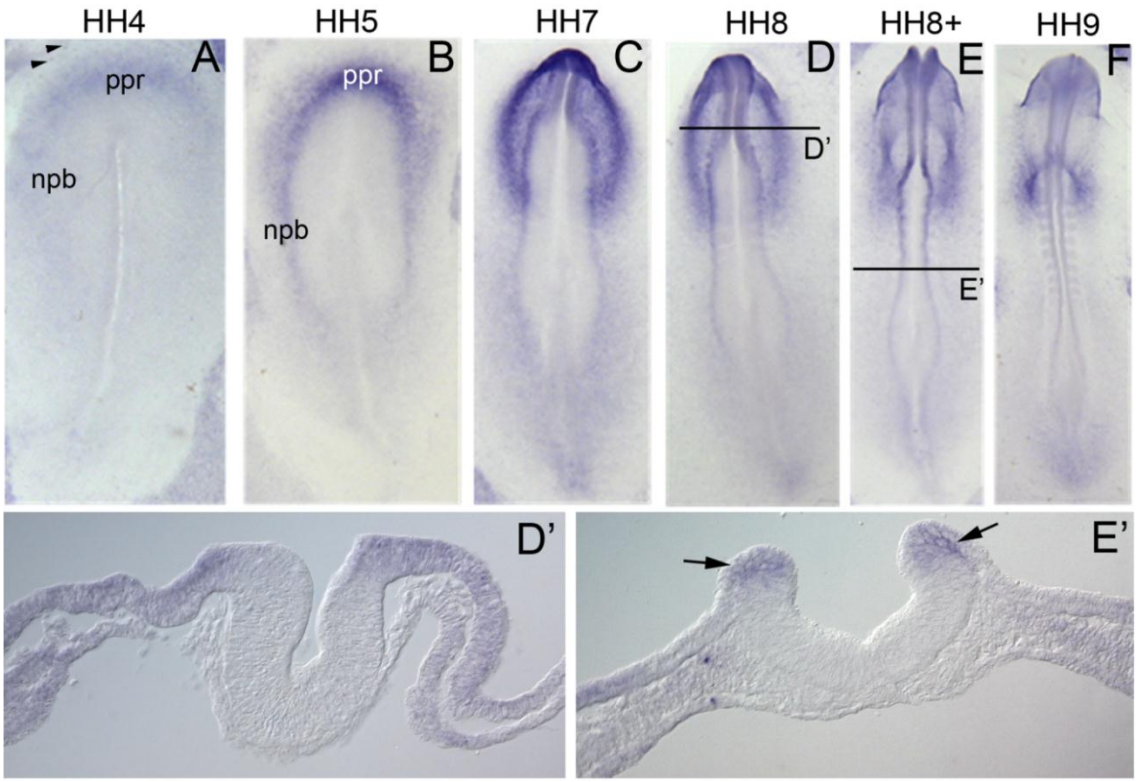


Figure 6: Expression pattern of *Prdm1* mRNA during early chicken embryogenesis. *Prdm1* is expressed in the neural plate border (npb) (A-E, arrow in E'), pre-placodal region (ppr) (A, B) and head ectoderm (C, D), and individual cells within the germinal crescent (A, arrowheads). (Nikitina, preliminary data)

When Prdm1 MO was injected, a delay in migration of the NC cells was observed as indicated by the shorter arrow pointing to the left, while normal migration was observed in the side that was not injected (Fig. 7). The use of Control MO did not cause any significant changes in the migration as seen in the bottom image (Fig. 7). This data supports the role of Prdm1 in NC EMT. Further experimentation is however required to validate this preliminary data and to explore the Prdm1 mechanism of action in the NC.

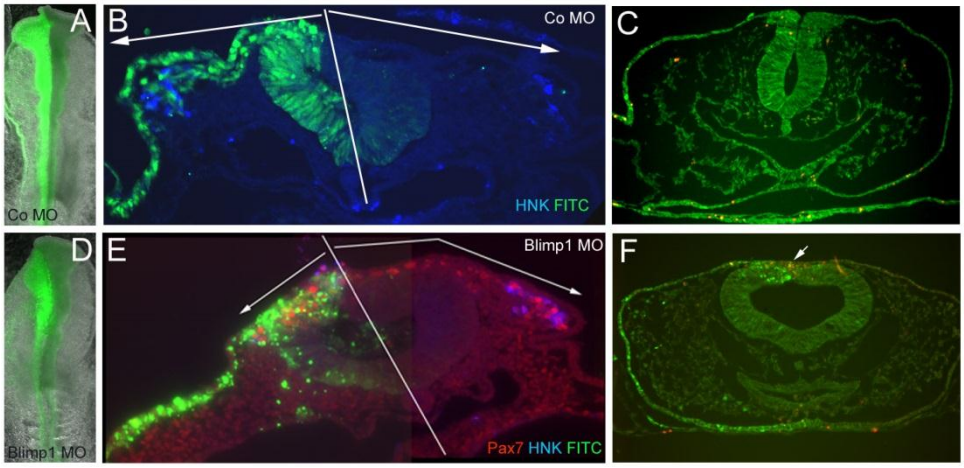


Figure 7: FITC-labeled Prdm1 morpholino oligonucleotides (MO) were used to disrupt translation of Prdm1 during neural crest development. Embryos were electroporated on one side with a control (A, B, C) or Prdm1-targeting morpholino (D, E, F). Panels A and D show whole embryo views with the left side incorporating the morpholino. In B and E the sections through the Control and Prdm1-electroporated emrbyos were stained with antibodies for Pax7 and HNk to visualize the neural crest. Arrows show the migration distances of the electroporated neural crest cells. The right side of the two neural tubes (A&D) was not injected and this side was used as a control to check migration of cells. (C and F) Tunel assay for cell death demonstrating increased cell death in Prdm1-morpholino electroporated neural crest (small arrow in F), but not in other regions of the embryo (Nikitina, unpublished data).

1.7. Hypothesis and Aims

We hypothesise that Prdm1 plays a role in the development of NC during chick embryogenesis and this is supported by the expression in the neural plate border (Fig.6). The question is does this role involve repression or activation of a set of genes? Does this repression happen in a direct manner or does it recruit co – repressors to the target DNA? Thus the first aim of this study involved exploring the expression of the known Prmd1 binding partners during NC development in the chick embryo by *in situ* hybridization. The second objective was to confirm the co-expression of Prdm1 and the binding partner(s) in chick neural crest by double *in situ* hybridization during early developmental stages.

CHAPTER 2

2. Materials and Methods

2.1. Eggs and Embryo

Un-incubated fertilized chicken eggs (*Gallus gallus domesticus)* were obtained from the Agricultural Research Council farm (Irene, Pretoria). This work has been approved by the University of Witwatersrand Ethics committee (the ethics approval number is 2014/04/O).

2.2 RNA isolation

Fertilized eggs were incubated at 37^0C until a desired stage (according to the Hamilton-Hamburger staging system) (Hamburger & Hamilton, 1992) was reached. After the embryos developed to the desired stage, the eggs were dissected and embryos homogenized in order to isolate total RNA using a SV RNA isolation kit (Promega). RNA was quantified and samples ran on agarose gel to confirm that the isolation was successful. Gene specific primers for individual candidate binding partners were used to synthesize cDNA and for amplification using PCR.

2.3. Primer design

Sequences for all of the binding partners were retrieved from NCBI nucleotide database. Gene specific primers for each respective binding partner were designed using Primer 3 program and then sent for synthesis at Inqaba Biotech. Before designing the primers the sequences were blasted to check for highly conserved regions, and then these regions were excluded to ensure high specificity of the resulting probe. To verify that genomic DNA was not amplified, primers were designed across exon-exon boundaries.

Table 2: The accession numbers of Prdm1 binding partners used in this work

Gene name	Accession number
TLE1	XM_003643034
TLE3	NM_001083927
TLE4	NM_204237
LSD1A/KDM1A	NW_003764157
G9a	X69838
HDAC1	AF_039751
HDAC2	NM_204831
Prmt5	XM_004950376

Annotated mRNA sequences for G9a and Tle3 (Table 2) could not be identified; therefore a different approach was used. mRNA sequences of G9a and TLE3 for other vertebrates e.g. mouse, human and zebrafish were obtained from the NCBI Nucleotide database. A pairwise sequence alignment of these mRNA sequences was performed to find regions of homology. A BLAST search of a highly conserved region was then performed on the whole chicken genome. Once chicken homologues of G9a and Tle3 were located in the chicken genome, primers were designed to amplify those sequences. For TLE 3, multiple splice forms have been identified in the chicken transcriptome. Primers were designed to amplify the region conserved in all three transcripts. All the primer sequences used in this work are included in Table 3. Old and new denotes the different primer sets tested. All the old primers were designed with a linker sequence at the beginning for restriction digest, and this was done because PCMV Sport 6 was used for cloning initially. The new primer sets were only designed if the old set did not work and in some cases all that had to be done was to remove the linker sequences at the beginning to ensure greater binding specificity.

Table 3: Primers that were designed for each Prdm1 binding partner

Gene name	Set of primers		Fragment size	
	Old	New	Old	New
TLE 1	F(EcoR1):TATGAATTCAAGGCAACATCCAGCAACTT R(XhoI): TATCTCGAGTTTCCTGCAGCACAAGACAG		618bp	
TLE 3	F(SalI): TATGTCGACCAGACATGGCACACACACAC R(XhoI): TATCTCGAGGGCTCCCTGAGCTGAATTTT	F:GTTTTAGGGTTTGGGGCTGT R:GGACGACTTCACCAAGAGGA	735 bp	413bp
TLE 4	F(EcoR1):TATGAATTCAAATGCTGATCGCAGTTGTG R(XhoI): TATCTCGAGCGGGCCAGACAGCTTTATTT		236bp	
HDAC 1	F(EcoR1):TATGAATTCGAGTCAATCTGCCCAAGAGC R (XhoI): TATCTCGAGAGTGAGACGCCACTCAGGTT	F: GAGTCAATCTGCCCAAGAGC R: AGTGAGACGCCACTCAGGTT	881bp	881bp
HDAC 2	F(EcoR1):TATGAATTCACAAGAAAGGAGCAAAGAAAGC R(XhoI): TATCTCGAGCAGCTCAGGAAAGGCAAATTT		449bp	
LSD1	F(EcoR1): TATGAATTCGCTTTCAGGAGCATTTGGAG R(XhoI): TATCTCGAGGAGCGAGAAGTGGAGAGTCC		809bp	
G9a	F(EcoR1): TATGAATTCAAACCAACGGTTCCTGTGAG R(XhoI): TATCTCGAGCTCAGAGTCGCCATCATCAA	F: TGCGCTGCTCCTATGATAAC R: AGGGGAGAACAACACCACG	1007bp	
Prmt5	F: CACGATCTTCCTCACCAACA R: CGCCAACTGGTGGAAGTTAT		841bp	

2.4. Reverse transcription (RT) and polymerase chain reaction (PCR)

Reverse transcription was performed using total RNA isolated from embryos at HH4-12. An H-minus Maxima reverse transcriptase enzyme (Thermo scientific) was used to catalyse the synthesis of the first cDNA strand. Immediately after the synthesis of the first strand a reaction was set up for the amplification of cDNA using a Ready Mix PCR enzyme from KAPA.

PCR reaction cycles included a 94^0C activation step, 94^0C denaturation, 51^0C (depending on Tm for primers) 72^0C extension and 72^0C elongation as well as optional 4^0C hold step. The 3 temperature cycles (94^0C, 51^9C and 72^0C) were repeated 35 times (MultiGene™ OptiMax Thermal Cycler). At the end of the reaction cycles, a 0.5% agarose gel was prepared and electrophoresis done to view the PCR products which were ran along with a 1kb gene ruler ladder (Thermo Scientific) to estimate size. The PCR products were then purified using a PCR clean up kit (Thermo Scientific) for downstream applications.

2.5. Cloning

A pGEM-T Easy vector kit (Promega) was used for cloning; this kit is supplied with competent cells (JM109) for efficiency of cloning. Before cloning the PCR products had to be quantified and volume needed for the ligation reaction calculated in accordance with the 3:1 ratio of vector (Fig.8) to insert. This ligation reaction was then incubated at room temperature for 1hour or in some instances overnight at 40^0C to obtain maximum number of transformants. Competent cells were then thawed on ice and mixed with the ligation mixture followed by heat shock at 42^0C. S. O. C medium (Appendix 1A) was then added to the mixture which was incubated further at 37^0C with shaking to allow bacteria to divide and replicate for 1 hour and 30 minutes. After this step the bacterial cells were spread on agar plate's containing ampicillin and incubated overnight at 37^0C. Colonies that grew on the agar plates were selected and grown in Luria broth (LB) containing ampicillin as pre-cultures. These pre-cultures were used to isolate and purify plasmid DNA.

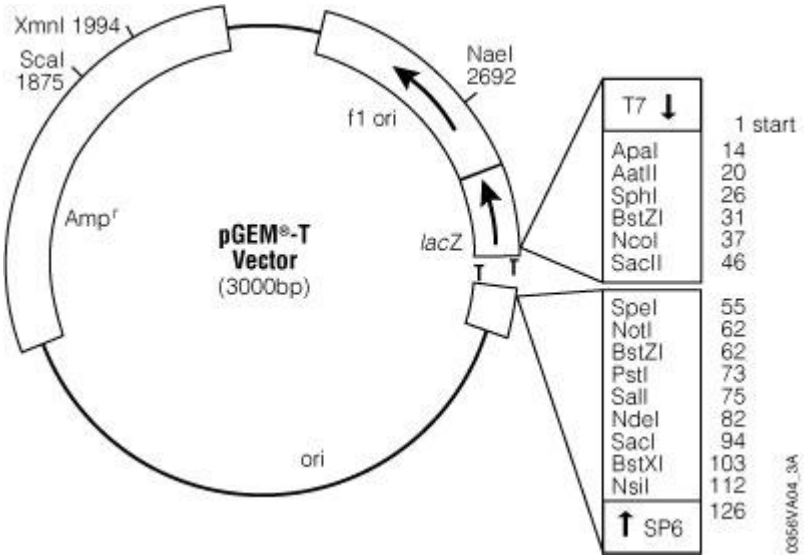


Figure 8: pGEMT – Easy vector map, with the T7 promoter and SP6 promoter site for in vitro transcription, restriction sites for making the vector linear

2.6. Purification of plasmids and sequencing

Plasmid DNA was purified using a Gene Jet plasmid purification kit (Thermo-Scientific) and the purified DNA was eluted in 50µl. The purified plasmid DNA was then enzymatically digested with *EcoRI* to remove the insert and assessed on an agarose gel before sequencing. All the samples that appeared to have inserts of the expected size were then sent for sequencing. Sequencing was done by Inqaba Biotechnology, and the results were sent back as chromatograms. These results were viewed using Finch TV which opened them in a Fasta format. Analysis was done by also performing a Blast search using the NCBI database. This was done to verify the sequences inserted in the plasmid as well as to determine the direction of the insert in order to use a correct RNA polymerase for *in vitro* RNA probe synthesis. After the sequence verification was done, the next step was to linearize the plasmid.

2.7. Plasmid linearization

Circular Plasmid DNA was then linearized and purified in preparation for *in vitro* transcription of RNA probes. *SalI* or *NcoI* was used depending on the direction of the insert. If the insert was in the forward direction from the T7 promoter, *SalI* was used to cut the plasmid. If it was in the reverse direction, then *NcoI* was used. The

reaction will all the reagents (Table 3) was incubated at 37^0C overnight, and if a fast digest enzyme was allowed to proceed for only 30 minutes to 1 hour.

Table 4: Reagents used in linearizing plasmids reaction

Reagents	Volume (µl)
SalI/*NcoI* enzyme	2
Appropriate Buffer	10
Plasmid DNA	To be calculated (10ng)
Water	To be Calculated
Total	100

2.8. RNA probe synthesis

The linearized clones inserted into pGEMT easy vector (Fig. 8) were used as templates to transcribe RNA using a T7 RNA polymerase as well a mixture of ribonucleotides including DIG labelled UTP (Roche). For sequences that were in the opposite direction SP6 RNA polymerase was used. The transcription reaction was incubated at 37^0C for 2 hours with extra T7/SP6 RNA polymerase added after 1 hour (Table 5). After the reaction was completed an agarose gel was ran to view products. The prepared probe was treated with DNAse I to degrade the template DNA and the reaction stopped by adding 0.5M EDTA. Another agarose gel was run to examine the results (RNA band only). Then the purification of RNA was done using a nucleospin RNA purification kit (Macherey-Nagel). Purified RNA ribo-probes were then stored in hybridization mix at -20^0C.

Table 5: Reagents used in synthesising DIG labelled RNA probes

Reagent	Volume (µl)
Linearized plasmid (1µg in DEPC ddH_2o)	To be calculated (1ng)
Transcription buffer	4
10x DIG/Fluorescein RNA labelling mix	2
RNAsin	0.5
T7/SP6 RNA polymerase	1.5
DEPC-treated water	To be calculated
TOTAL	20

2.9. *In situ* hybridization

Fertilized eggs were obtained and incubated to HH4-HH12. After the embryos had developed to the desired stage, they were dissected out of the eggs and washed briefly in Ringers solution and then fixed overnight. These were the steps that were followed to fix and dehydrate the embryos for storage. The dehydration step is important to prevent RNA degradation during storage.

1. Embryos were collected and left in 4% PFA @ 4^0C overnight
2. The following day 4% PFA solution was taken off
3. Diethylpyrocarbonate (DEPC) treated-PBT solution was added-this was placed on the nutator for 5-15mins. The solution was discarded and the step was repeated three times
4. To dehydrate embryos – embryos were placed in a methanol series for 15mins on nutator. Where 25% Methanol (MeOH) + PBT/DEPC was added first, followed by 50% MeOH + PBT/DEPC and then 75% MeOH + PBT/DEPC.

5. Then finally they were washed with 100% MeOH while shaking for 15mins, this step was done twice before the embryos were stored in methanol (100%) at -20^{0}C.

Before using the embryos for hybridization they had to be rehydrated using a methanol series until they were in PBT/DEPC. Hybridization was done according to the protocol: *In situ* hybridization analysis of chick embryos in whole mount and tissue sections (Ausubel et al., 2002), with a few modifications detailed below. The embryos were fixed in 4% paraformaldehyde (PFA) at 4^{0}C overnight for preserving the morphology and to cross link the proteins to ensure that the tissue is less fragile.

After cross linking they were dehydrated in methanol/PBT series until 100 % methanol was reached. Before the hybridization steps the embryos were rehydrated again in the methanol series with PBT, treated with proteinase K. After the treatment with proteinase K, they were washed with probe and left to hybridize overnight at 70^{0}C in a hybridization oven (Memmert).

Probe was removed and stored in a hybridization mix, and 3%BSA was used to block non-specific site and an anti-digoxigenin (DIG) antibody conjugated to alkaline phosphatase (AP) (Roche) was used to detect probes. To detect the signals, the NBT/BCIP substrate (Roche) was used. Successive washing steps with MABT were required to remove unbound antibody. Embryos were fixed again in 4% PFA to destroy the active site of AP and embryos are dehydrated to remove background staining and photographed using Nikon SMZ 1500 stereomicroscope.

2.9.1. Double *in situ* hybridization

Double *in situ* hybridization is a technique the uses 2 probes simultaneously and takes bit longer to perform because these two probes have to be detected sequentially. Embryos from stages HH4 – HH7 were prepared and probed with a combination of FITC-labelled Prmd1 probe and each of the following DIG-labelled probes: *G9a, Lsd1, Hdac1, Hdac2, Prmt5* and *Tle3* (Table 6). Anti-FITC antibody followed by NBT/BCIP was used to detect the weaker probe, which was *Prmd1*.

NBT/BCIP is a colour reagent that produces a purple colour precipitate. This was allowed to develop for about 5 days and then embryos were washed and a second colour reagent, INT/BCIP, was used to detect the other probes (it produces a light brown colour). NBT/BCIP and INT/BCIP are both colour substrates that are hydrolyzed by the enzyme alkaline phosphatase which is conjugated to the antibody. The area of mRNA co-localization would have purple and brown colour to show co-expression. After this step embryos were not washed with ethanol (because INT/BCIP produces precipitate that is soluble in alcohol) but were processed with PBT and then glycerol and were ready for photographing.

Table 6: The list of RNA probes that were used for *in situ* hybridization

Probe name	**Length of insert (bp)**	**Linearized with enzyme**	**Transcribed with (T7 or SP6)**	**Source**
G9a	1007	*SalI*	T7	This work
Tle1	618	*SalI*	T7	This work
Tle3	413	*SalI*	T7	This work
Hdac1	881	*SalI*	T7	This work
Hdac2	449	*SalI*	T7	This work
Lsd1	809	*NcoI*	SP6	This work
Prmt5	841	*SalI*	T7	This work
Prdm1	660	*SalI*	T7	Nikitina, unpublished data
Snail2	1500	*EcoRI*	T7	Bronner lab, Caltech

2.9.2. Photographing

Stained embryos were dehydrated and stored in 100% methanol. Before photographing, embryos were first rehydrated using methanol series until they could be washed in PBT. 25% and then 50% glycerol mixed with PBT was made to wash the embryos before viewing. 30% glycerol was used to view and photograph the stained embryos. Embryos were placed in a Petri dish containing a very thin layer of 0.5% polymerised agarose. After photographing, embryos were stored in 30% glycerol mixed with PBT. At first a Zeiss AxioCam stereomicroscope was used, and then Nikon SMZ 1500 stereomicroscope was used with a DS- Fi1 camera to view and take photographs of the embryos. All the photographs were saved as TIFs and further processed using Elements 12 Photoshop.

2.10. Cryo-sectioning

Embryos stored in 30% glycerol were processed by first washing in PBT to remove glycerol. They were further cryoprotected in 15 % sucrose for 5 hours and then in 30% sucrose overnight in preparation for embedding. Clear cryomatrix (Labotec) was used for embedding, and because chick embryos are fragile they were placed in a Petri dish and covered with the cryomatrix. A rubber mould was used to individually embed each embryo by flash freezing in liquid nitrogen, the samples were then stored at -20 ^{0}C. Sections were obtained by using a Leica CM 1510 S cryostat which was set to slice at 20 microns. Slides with sections were processed by washing in PBS at 42^{0}C for 10 minutes and then further washed in PBS at room temperature twice for 5 minutes. Before they were mounted using cc mounting medium (Sigma), excess PBS had to be wiped. Slides were covered with cover slips immediately after putting drops of mounting medium and were left to dry up overnight. Photographs of these sections were then taken using the Olympus Provis Ax microscope and images were processed using Elements 12 Photoshop.

CHAPTER 3

3. Results

3.1. Preparation of Prdm1 probe templates and RNA probes.

Cloning of the probe templates for G9a, Hdac2, and Tle1 was accomplished successfully during my Honours project (Thembekile Zwane, honours project). Probe templates for – Hadc1, Lsd1, Tle3 and Prmt5 were successfully prepared as part of this project.

RNA was isolated from embryos incubated to HH9 and the integrity and quantity of the RNA checked by electrophoresis on 0.5% agarose gel (Fig. 9) PCR amplification was successful for Hdac2, Tle4, Tle1 and G9a (Fig. 10A). However, in order to obtain successful amplification of Hdac1, Lsd1, Prmt5 and Tle3 we had to optimize the PCR conditions. Prmt5 is another binding partner which was added to the list. This was done by first altering the annealing temperature and in cases where this was not successful, we then had to lower the concentration of primers used in the reaction (Fig. 10B). We obtained the fragments of desired size (see gels), and cloned them into pGEM-T Easy and selected clones that contained the insert of desired size (see a representative gel in the Appendix Fig.23). Three individual clones for each probe template were sent for sequencing; those that contained the correct sequence were used to prepare RNA probes. Gels showing successful probe preparation are shown in Figure 11A-C for all the binding partners.

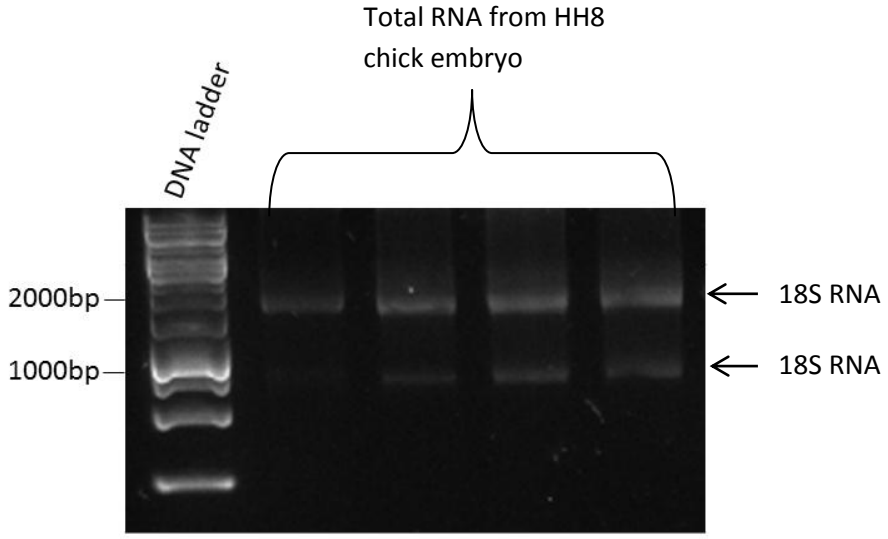


Figure 9: Agarose gel showing the results of RNA extraction from 8 chick embryos, 1kb DNA ladder is included to indicate band size.

Three members of this family of proteins namely Tle1, 3 and 4 were chosen for analysis. All 3 of them were cloned successfully and sequenced, however only Tle1 and 3 worked. Tle3 first gave multiple fragments and none of the fragments were of the expected correct size. To get the correct fragment, trouble shooting was done. First the annealing temperature was modified to get a specific DNA band, however this did not give significant results. Secondly primer concentration was deceased, however multiple bands were still observed. This led to the speculation that the DNA degradation step was not performed correctly. New DNAse as well as buffer was used to degrade any DNA present in the RNA samples

After trying different troubleshooting methods and they did not work, we decided to re-design the primers. A new set of primers were designed and synthesised (Table 3). DNA fragment of about 436 bp was successfully amplified and cloned (Fig.10B).

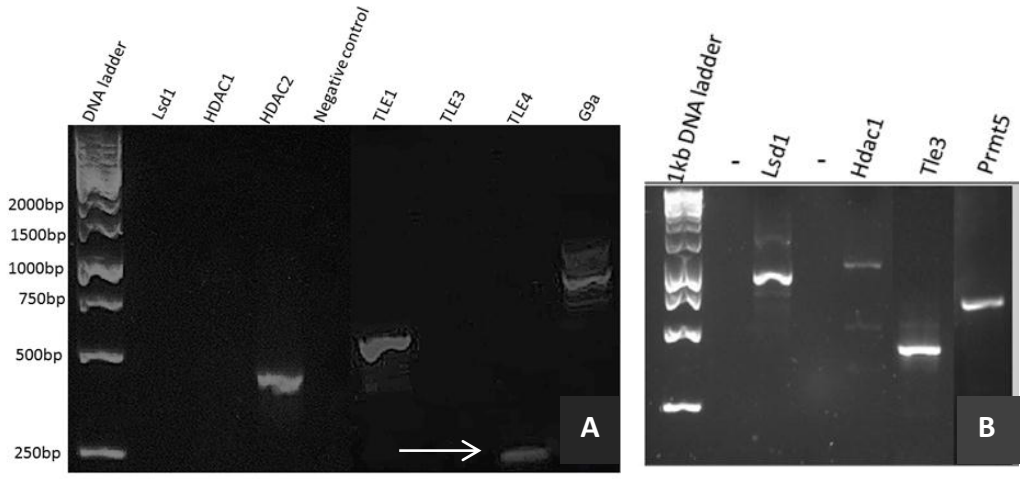


Figure 10: Results of PCR amplification of the 7 binding partners shown on the gel after electrophoresis including those that never worked the first time. Tle4 had the smallest fragment therefore the band ran lower on the gel (arrow). The PCR products were run on 2 separate gels but the images of the gels were merged for space.

RNA probes were successfully transcribed and the DNA fragment was removed using DNAse in order to eliminate interference upon hybridization. These probes were then cleaned up to remove all the other reagents used in the synthesis and RNA was mixed with hybridization mix and stored.

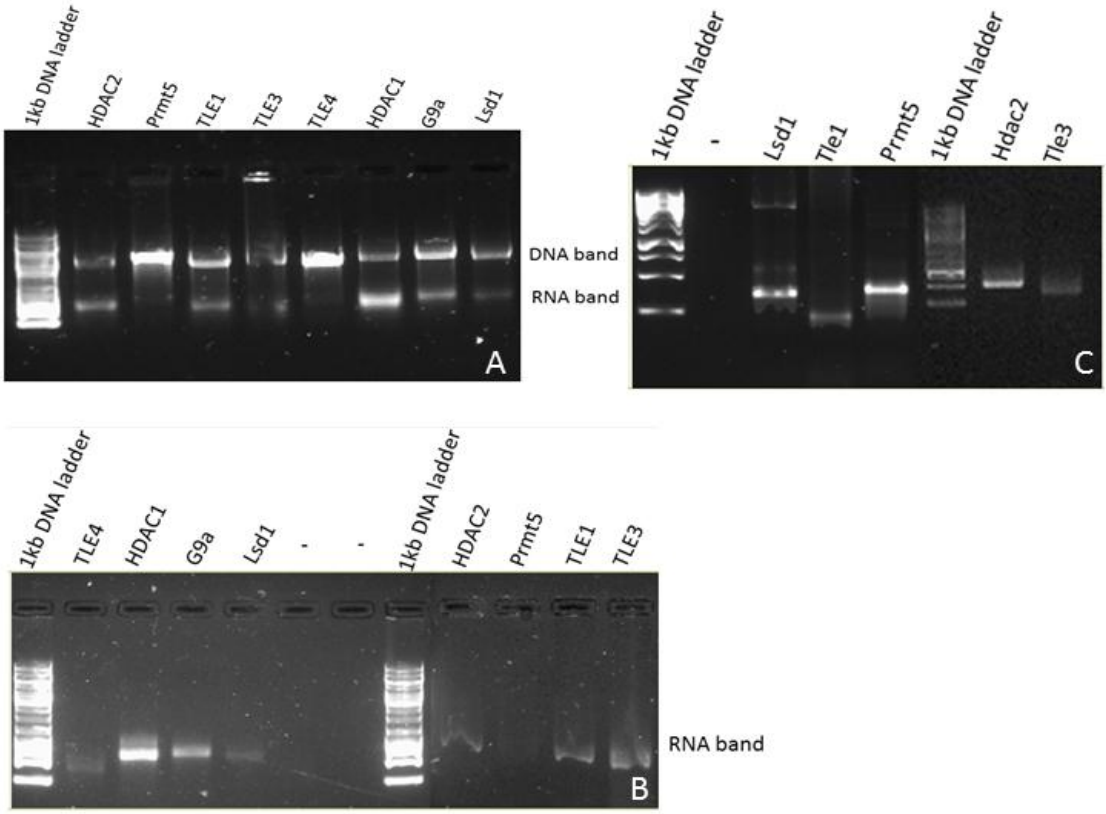


Figure 11: **RNA probe synthesis before DNA degradation step (A) and after DNAse treatment (B) ran on agarose gel. The samples were loaded as indicated by the labels and the (-) indicates that no samples were loaded in those wells. RNA probe synthesis of the probes that did not work as well as those that had faint bands is shown in gel C.**

Probe synthesis yielded the results observed in Figure 11 (A-B) and, though *Tle3*, *Tle4* and *Prmt5* did not synthesize successfully this was repeated and RNA probes were obtained. *Tle1* and *Hdac2* were also newly synthesised since they had faint bands (Fig. 11B), and the gel in Figure 11C shows results after DNAse treatment.

All probe templates for Prdm1 binding partners were used for *in situ* hybridization on whole chick embryos at HH4-HH12. Sense probes were prepared for each gene and used as a negative control for *in situ* hybridization specificity. For some genes, double *in situ* hybridizations were performed with *Snail2* probe to confirm expression in the premigratory and migrating neural crest cells.

3.2. *Prdm1* is expressed in NPB and in the otic placodes

In order to gain better insight into the expression pattern of *Prdm1* we performed additional *in situ* hybridization and sectioning. In addition to observed expression in the neural plate border and the preplacodal domain, we also observed expression in the otic placodes (Fig. 12 D''', D''''), head ectoderm and the foregut. *Prdm1* mRNA continues to be present in the neural tube until HH10+, though at that time its expression in the dorsal neural tube is reduced to a small region overlapping with the midbrain-hindbrain boundary (Fig. 12G, G'' asterisk). As the cranial NC cells start to delaminate and migrate, *Prdm1* expression is turned off in the NC cells and in the neural plate border, however *Prdm1* persists in the otic placodes (Fig. 12F, G, G'''). Double *in situ* hybridization with *Snail2* confirmed co-expression of this marker with *Prdm1* in the premigratory neural crest (Fig. 12J, K &K').

Whole mount pictures of the double *in situs* of some binding partners (*Prdm1*, *Tle3*, *Hdac1*, *Hdac2* and *Lsd1*) with *Snail2* were included as a separate figure in the appendix (Fig. 22). This shows the expression of these particular binding partners with *Snail2* at stages between HH8 and HH11. Some of the images show crystals that settled on top of the embryos and could not be removed. This was a result of longer incubation time in the INT/BCIP solution. Because the co-expression (or lack thereof) of the binding partners with *Snail2* could be better seen in sections, we decided to show the whole mount images in the appendix only and not the main figures.

3.3. *G9a* is expressed in the neural plate, neural tube, and the neural plate border

G9a expression starts in the anterior epiblast at HH4 (Fig. 13A). At HH5 and 6 the expression spreads throughout the epiblast but is turned off in the Hensen's node and primitive streak (Fig. 13B, C, and C"). The expression of *G9a* mRNA is seen throughout the neural plate at HH6 to HH8- (Fig. 13C-E). At HH8-, *G9a* is expressed throughout the neural plate, but not in the non-neural ectoderm; somites and mesoderm also show some *G9a* expression (Fig. 13E, E''').

By HH9-, *G9a* expression is seen posteriorly in the open neural plate (Fig. 13F), at the trunk level in the somites, lateral plate mesoderm, notochord and the neural tube, but not in the non-neural ectoderm or endoderm (Fig. 13F, F", F''', G). More anteriorly, *G9a* is expressed in the tip of the neural tube/pre-migratory neural crest, as well as in the dorsal NT (Fig. 13F'). At HH9, the region with the most prominent expression is the midbrain-hindbrain boundary (Fig. 13G). By HH11, *G9a* expression is turned off throughout the embryo, with the exception of a small area of strong expression in the open neural plate (Fig. 13H, asterisk).

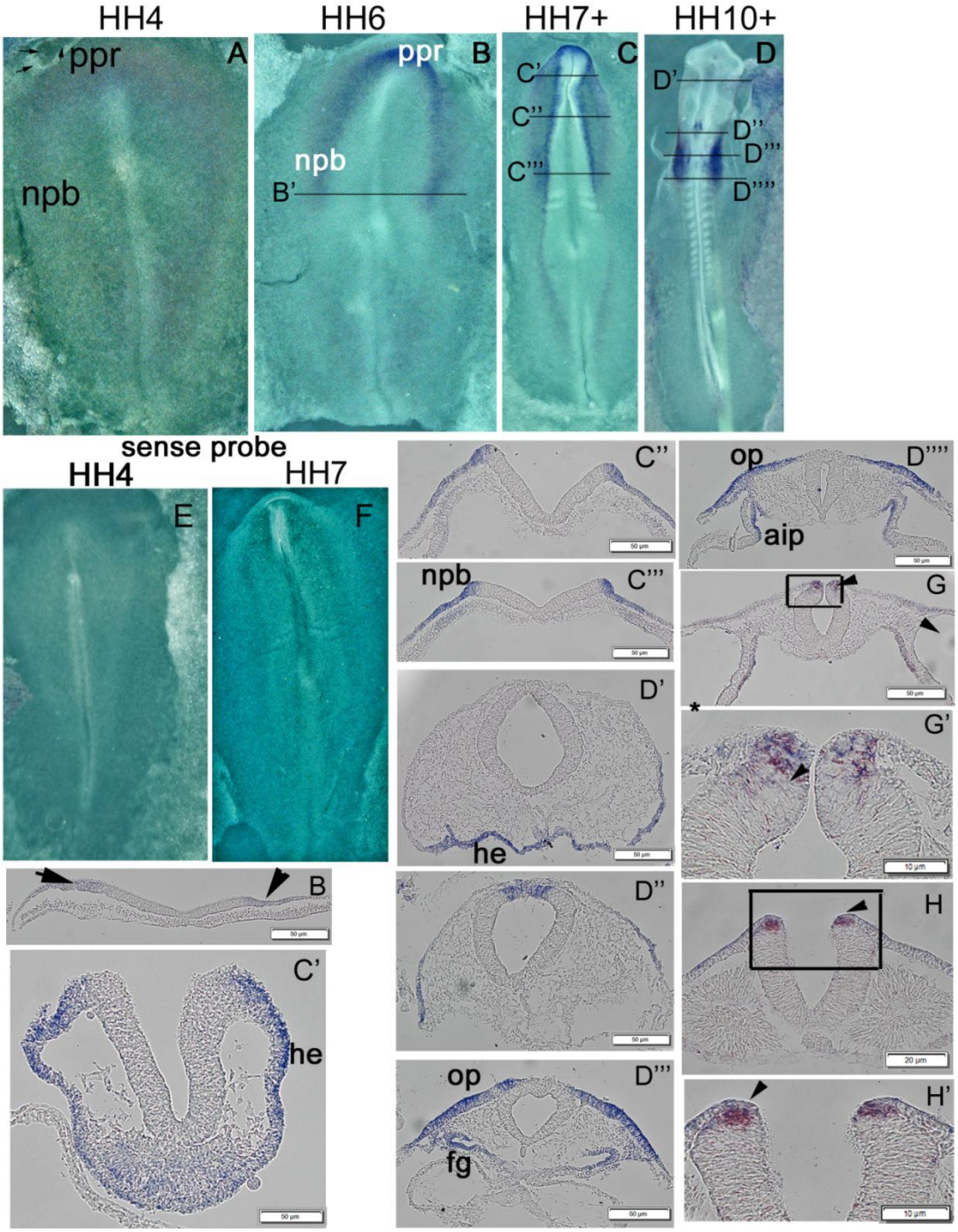


Figure 12: Additional data on *Prdm1* expression at a later stage (HH10+), with more sections showing expression on the neural plate border (npb) (A-B, B', C'''), pre-placodal region (ppr) (A, B), head ectoderm (C, C''), ear placodes (D''', D'''') and anterior intestinal portal/foregut (D''', D''''). (E-F) – HH4 and HH7 embryos hybridized with the sense probe: double *in situ* hybridization for *Snail2* (brown) and Prdm1 (purple) of an HH8+ embryo, arrowheads indicate co-expression in the premigratory neural crest. (G') shows a higher magnification of the region boxed in (G), and (H') – the region boxed in (H). Npb- neural plate border, ppr – preplacodal region, he – head ectoderm, aip –anterior intestinal portal, fg- foregut, m - mesoderm. Scale bars: B-D''''' 50µm, G- H'20µm.

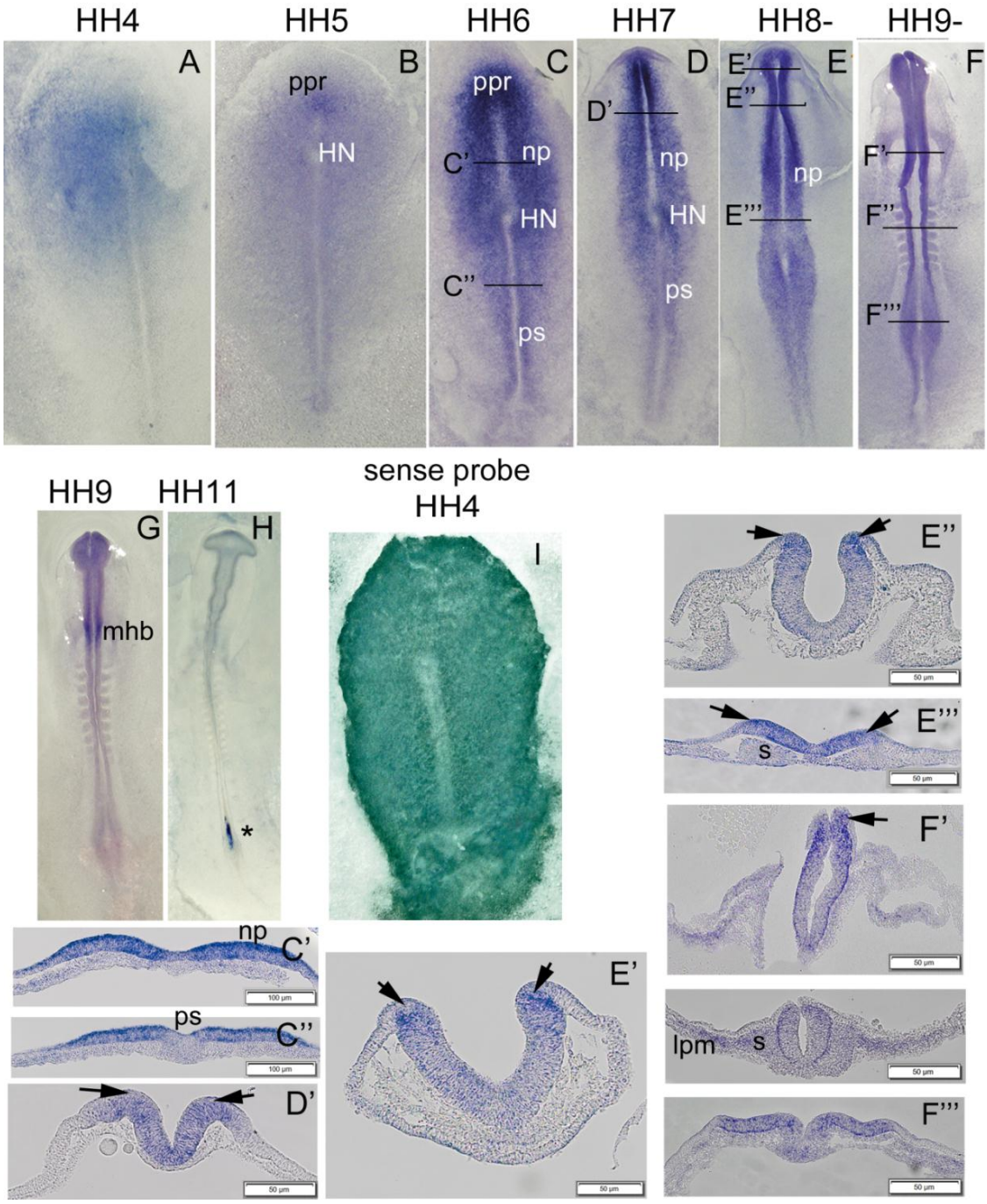


Figure 13: Expression of *G9a* mRNA in stage HH4-HH9 chicken embryos *G9a* transcripts are seen in the epiblast (A-C), neural plate (C-F, C', D''), neural plate border (CF, arrowheads in D', E', E'', E'''), somites and lateral plate mesoderm (E''', F''). By HH11, the expression of *G9a* is reduced and appears to be restricted to a small region in the open neural plate (H, asterisk). (I) – HH4 embryo hybridized with the sense probe. HN- Hensen's node, np – neural plate, ps- primitive streak, ppr – preplacodal region, mhb – midbrain-hindbrain boundary, s– somites, lpm – lateral plate mesoderm. Scale bars: C', C'' - 100µm, E'-F'''- 50µm.

3.4. *Lsd1* expression is seen in the neural tube, neural plate border, premigratory and delaminating neural crest.

Lsd1 expression is first seen at Eyal-Giladi and Kochav stage XII (Eyal-Giladi & Kochav, 1976) in the centre of *zona pellucida* and extra-embryonically in future blood islands (Fig. 14A). *Lsd1* expression is almost ubiquitous at HH4-5, with higher levels of the transcript in the epiblast, and lower levels in the hypoblast and primitive streak (Fig. 14B-B', C). By HH6, the *Lsd1* mRNA becomes restricted to the neural plate and the future lateral plate mesoderm, while there is no *Lsd1* expression in the non-neural ectoderm or segmental plate mesoderm (Fig. 14D-D'). At HH7+-HH8+, *Lsd1* expression is seen in the neural plate, especially in the neural plate border (Fig. 14E', E'', F', arrows), dorsal somites and lateral plate mesoderm (Fig. 14F''), as well as in the lateral border of anterior intestinal portal (Fig. 14E'', F').

By HH12, *Lsd1* mRNA can be seen throughout the neural tube, in the otic placodes, dorsal somites and lateral plate mesoderm (Fig. 14H-H'', I). In order to find out if *Lsd1* is present in migrating neural crest cells, we performed double *in situs* for *Snail2* and *Lsd1* at HH10+. Our results showed co-expression of these two genes in the pre-migratory and delaminating cranial neural crest cells (Figure 14L-M') also refer to Appendix 2 (Fig. 22).

3.5. *Prmt5* is expressed in the neural plate/tube, neural plate border and non-neural ectoderm

Prmt5 expression commences at or before Eyal-Giladi and Kochav stage XII (Eyal-Giladi & Kochav, 1976) throughout the *zona pellucida*, with the exception of the very centre (pattern of expression opposite to that of *Lsd1*) (Fig. 15A, A'). During gastrulation, *Prmt5* is seen in both epiblast and hypoblast, but there appears to be lower levels in the hypoblast and in the primitive streak (Fig. 15B, C, and C'). At HH7/8 expression is seen in the neural plate, especially at the neural plate border (Fig. 15D, E). At HH8+-HH9, *Prmt5* transcripts can be observed in the neural plate/tube, non-neural ectoderm, anterior intestinal portal, segmental plate and lateral plate mesoderm (Fig. 15F, G and the sections thereof).

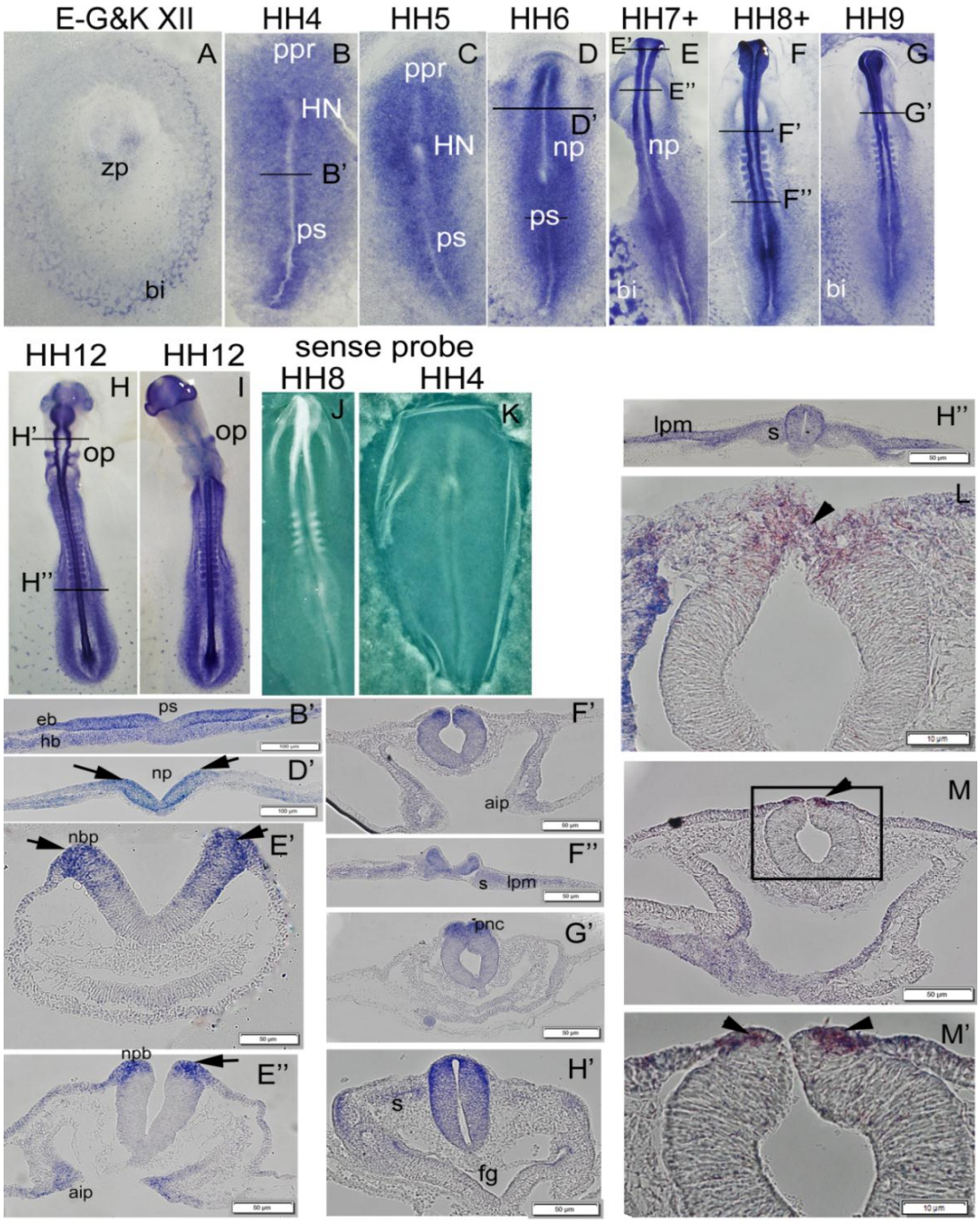


Figure 14: ***Lsd1*** **expression pattern in HH3-HH12 chicken embryos.** ***Lsd1*** **is widely expressed at early developmental stages (A-D).** ***Lsd1*** **expression becomes restricted to the neural plate/tube, otic placodes and mesoderm at later stages (E-I). (I) shows the same embryos as (H), ventral view. (J, K) – HH8 and HH4 embryos hybridized with the sense probe. (L-M') – double** ***in situ*** **hybridization for** ***Snail2*** **(brown) and** ***Lsd1*** **(purple) of an HH10+ embryo, arrowheads indicate co-expression in the pre-migratory and migrating neural crest cells. Eb – epiblast, hb – hypoblast, zp – zona pellucida, bi – blood islands, ps- primitive streak, HN – Hensen's node, ppr – preplacodal region, np-neural plate, npb- neural plate border, aip-anterior intestinal portal, fg – foregut, lpm – lateral plate mesoderm, s – somites, pnc – premigratory neural crest, op – otic placode. Scale bars: B'', D' - 100µm, E'-H'', M - 50µm, L, M' - 10µm.**

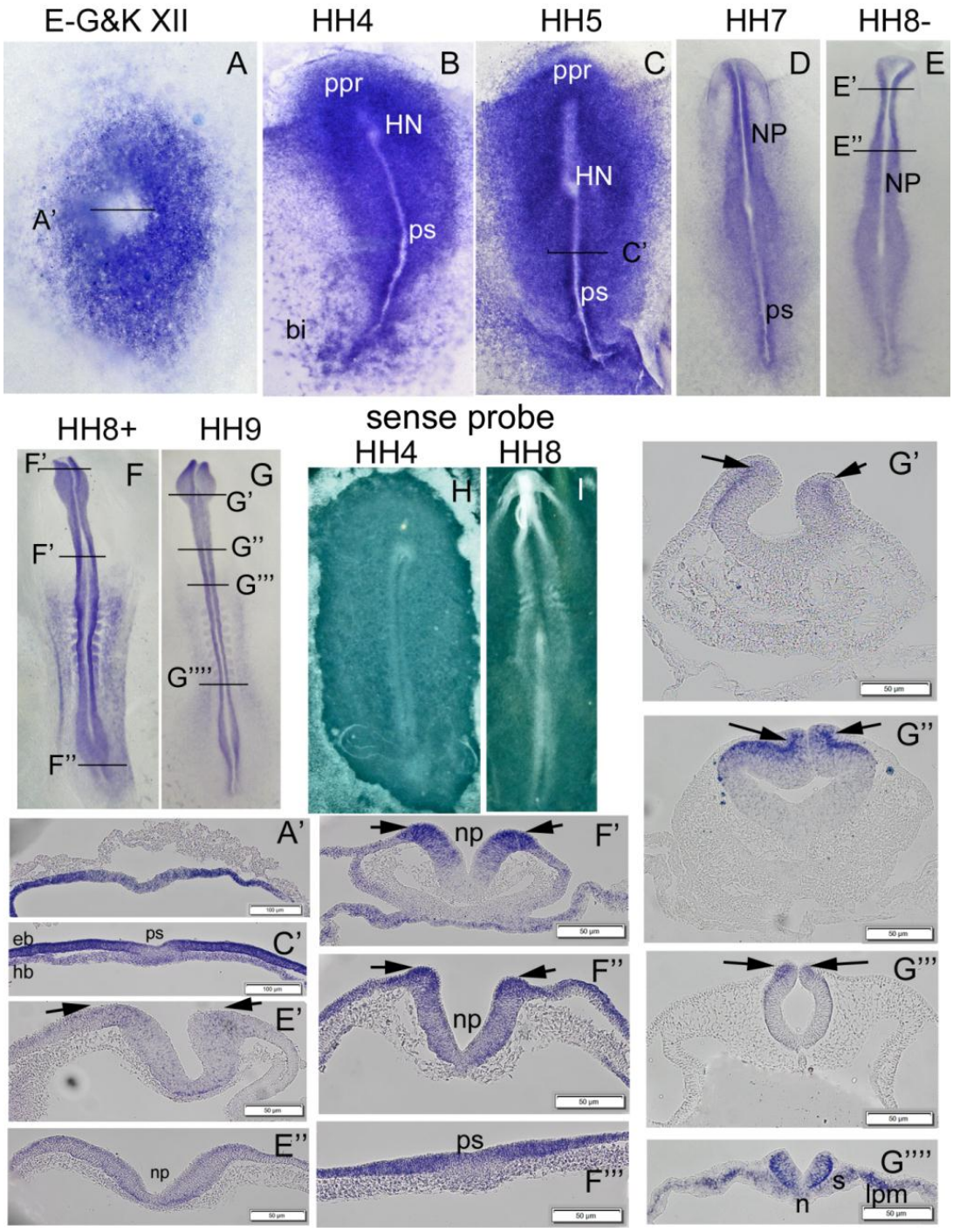


Figure 15: *Prmt5* is expression during early chicken embryogenesis. *Prmt5* is expressed in the epiblast (A-C, C'), neural plate/tube (D-G, E'-E'', F'-F'', G'-G''''), somites and lateral plate mesoderm (G''''). . (H, I) – HH4 and HH8 embryos hybridized with the sense probe. Eb – epiblast, hb – hypoblast, bi – blood islands, ps- primitive streak, HN – Hensen's node, ppr – preplacodal region, np-neural plate, lpm – lateral plate mesoderm, s – somites, n-notochord. Arrows indicate Prmt5 expression in the neural plate border. Scale bars: A', C' - 100µm, E'-G'''' - 50µm.

3.6. *Hdac1* expression is seen in the NPB and migrating NC

During gastrulation (HH4 and HH5) *Hdac1* transcripts are found throughout the epiblast, and in the extra embryonic tissues. However expression is not seen in the prospective mesoderm and endoderm cells migrating through the primitive streak (Fig. 16A-C, B'). At HH6 and HH7, *Hdac1* is expressed in the neural plate, as well as in the non-neural ectoderm, and epiblast (Fig. 16C, D). At HH8- and HH8, expression of *Hdac1* can be observed in the neural plate border, dorsal neural tube, non-neural ectoderm, somites, lateral plate mesoderm and anterior intestinal portal (Fig. 16E, F and sections thereof).By HH10-, *Hdac1* transcripts can also be seen in the migrating neural crest cells (Fig. 16G, asterisk, 16G'). Double *in situ* staining with *Snail2* demonstrates co-expression of *Snail2* and *Hdac1* in migrating neural crest cells (arrow in Fig. 16J') also see Appendix 2 Figure 22.

3.7. *Hdac2* is expressed in the NPB but not in NC

The expression of *Hdac2* is first seen during gastrulation, *Hdac2* transcripts are found in the epiblast, but in a more narrow area than *Hdac1* transcripts (Fig. 17A, B). Neither the primitive streak nor the ingressing prospective mesoderm cells express this transcript (Fig. 17B', red arrows). During neurulation, *Hdac2* is expressed throughout the neural plate including the neural plate border (Fig. 17C-E, D', E'-E'''). At HH10, expression of *Hdac2* is observed in the anterior intestinal portal (Fig. 17E'', 14F'''), otic placodes (Fig. 17F, F'') and non-neural ectoderm (Fig. 17F', 17I-I'). Double *in situ* hybridizations with *Snail2* at HH11 revealed that at that stage, there is no overlap between the expression domains of the two markers, with *Hdac2* expression being confined to the head non-neural ectoderm and absent from the neural crest or the dorsal neural tube (Fig. 17I-J and Appendix 2 Fig. 22). The summary of expression patterns is included in Table7.

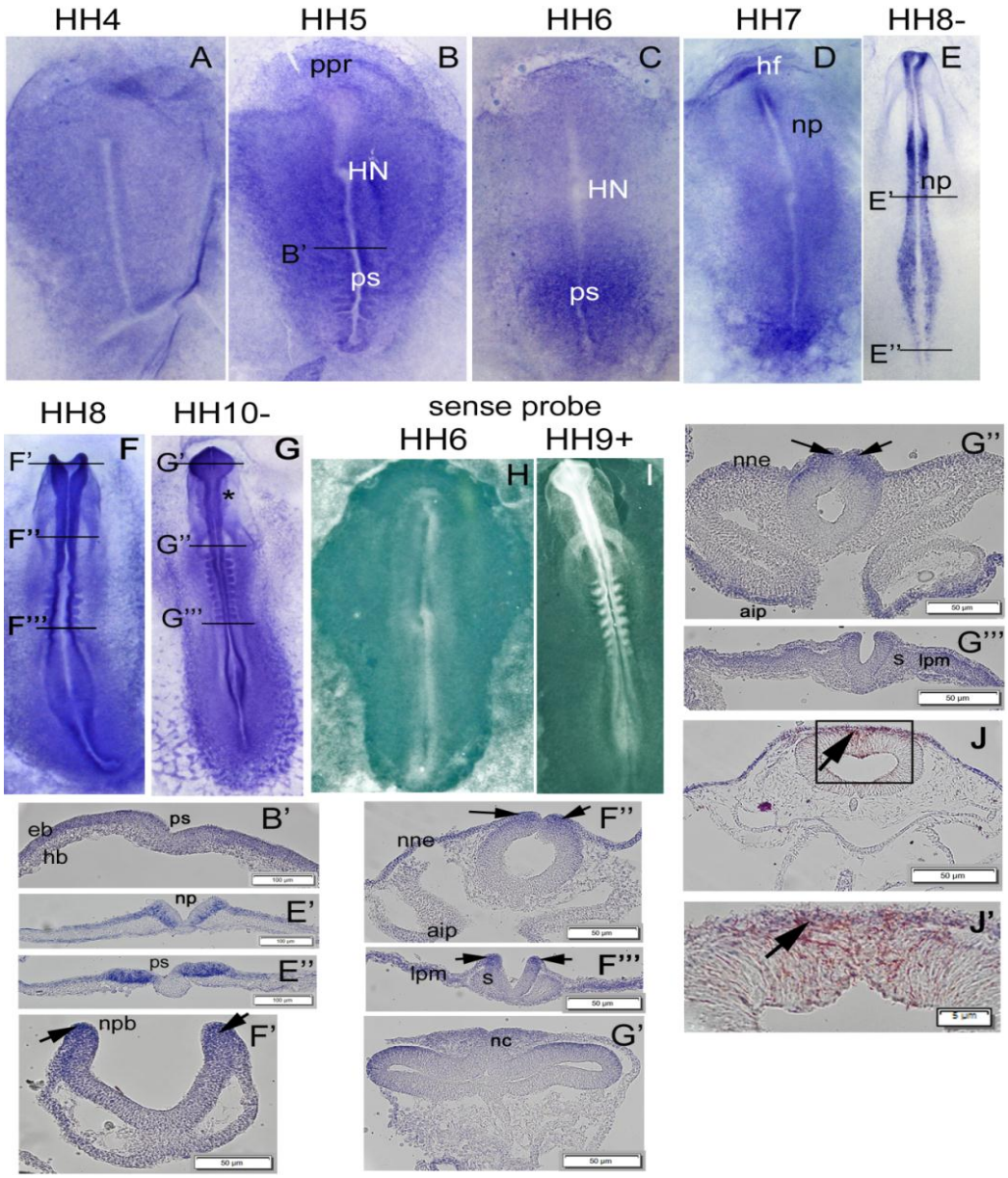


Figure 16: *Hdac1* expression in stage 4-9 chicken embryos. *Hdac1* exhibits expression in the epiblast (A-D), neural plate and neural tube (D-G), somites and lateral plate mesoderm (F''', G''), anterior intestinal portal (F'', G''), non-neural ectoderm (F, G, F'', G'') and migrating neural crest (G', J, J'). (H, I) HH6 and HH9+ embryos hybridized with the sense probe. (J-K): double *in situ* hybridization for *Snail2* (brown) and *Hdac1* (purple) of an HH10 embryo, arrowheads indicate co-expression in the migrating neural crest. (J') shows a higher magnification of the region boxed in (J). Ps- primitive streak, np – neural plate, eb- epiblast, hb – hypoblast, HN- Hensen's node, ppr- preplacodal region, aip-anterior intestinal portal, hf-head fold, s-somites, lpm-lateral plate mesoderm, nc-neural crest, npb – neural plate border, nne – non-neural ectoderm. Arrows in F', F'', F''', G'', G''' indicate expression in the neural plate border, arrows in F and J' indicate migrating neural crest cells co-expressing *Snail2* and *Hdac1* is observed in pre migratory as well as migrating neural crest as shown by the sections from double *in situ* hybridization with a neural crest marker *Snail2* (J). Scale bars: B'-E''-100µm, F'-J - 50µm, J'-5µm

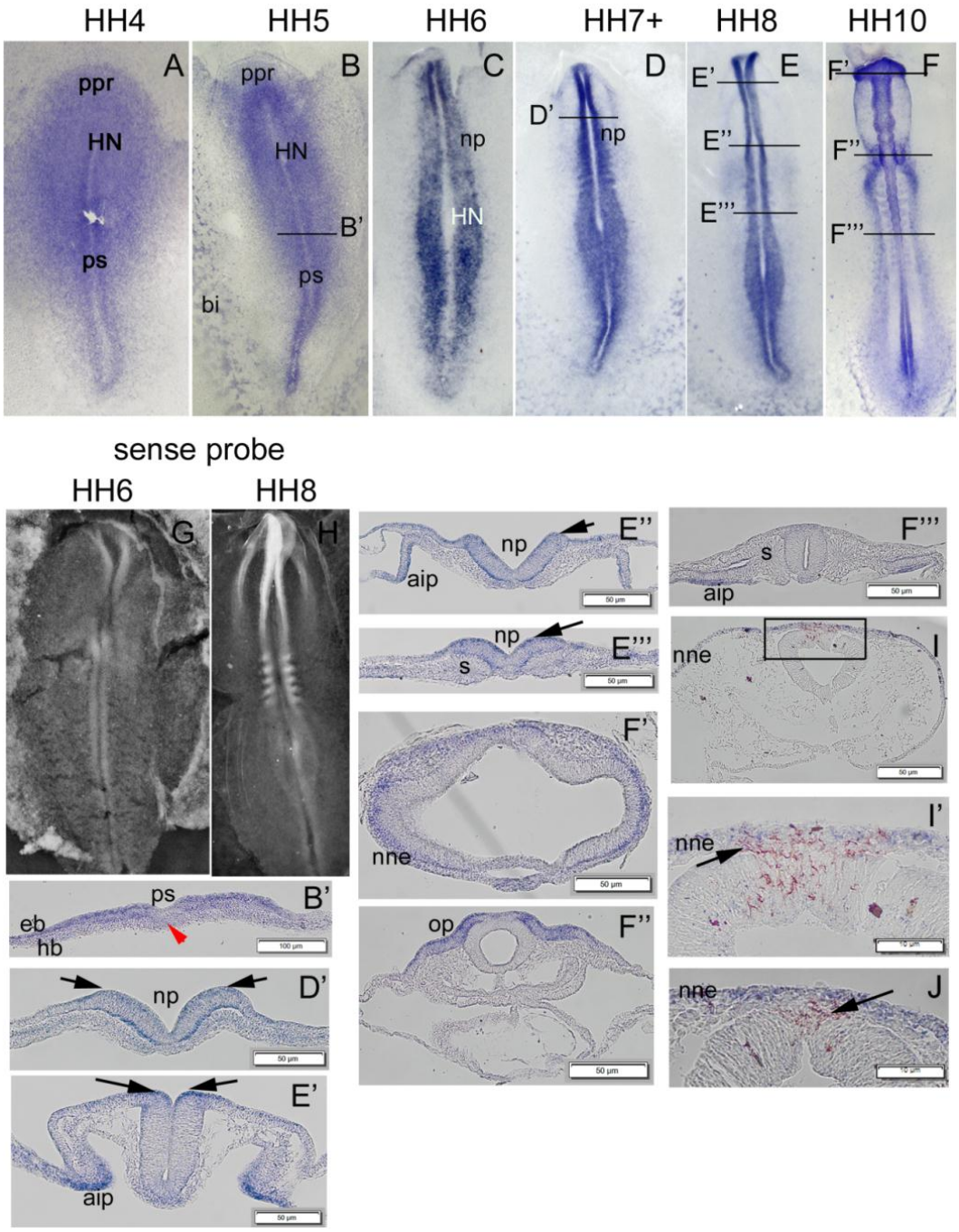


Figure 17: Expression pattern of *Hdac2* mRNA in HH4-HH11 chicken embryos. *Hdac2* is expressed in the epiblast (A-D), the neural plate/tube (C-F, C', D', E'-E'''), otic placodes (F''), cranial mesenchyme (F') and anterior intestinal portal (E''-F'''). (G, H) HH6 and HH8 embryos hybridized with the sense probe. (I-J): double *in situ* hybridization for *Snail2* (brown) and *Hdac2* (purple) of an HH11 embryo, arrowheads indicate *Snail2*-positive migrating neural crest cells. (I') shows a higher magnification of the region boxed in (I). Ps- primitive streak, HN- Hensen's node, eb -epiblast, hb- hypoblast, ppr- preplacodal region, np- neural plate, aip- anterior intestinal portal, s-somites, op- otic placode, nne – non-neural ectoderm. Arrows in D', E', E'' indicate expression in the neural plate border, arrows in I-J point at the delaminating neural crest which expresses *Snail2* but not *Hdac2*. Scale bars: B' - 100µm, D'-I - 50µm, I', J - 10µm.

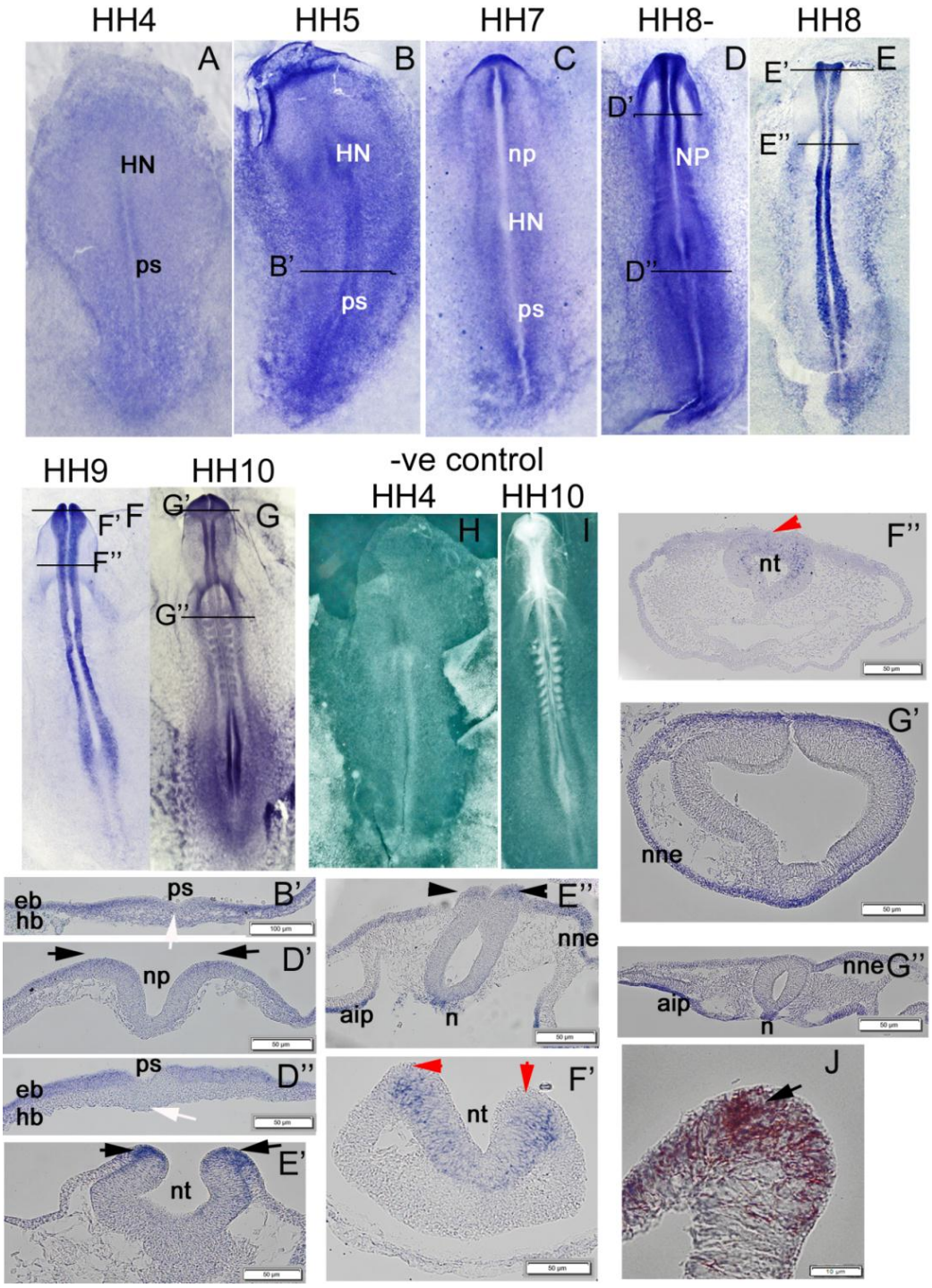


Figure 18: *Tle3* expression pattern during early chicken embryogenesis. *Tle3* is expressed throughout the epiblast (A-C, B', D'), in the neural plate with particularly strong expression in the neural plate border (C-F, D', E', E'', J, arrows), non-neural ectoderm (E'', G', G'') and anterior intestinal portal (E'', G''). (H, I) HH4 and HH10 embryos hybridized with the sense probe. (J) Double *in situ* hybridization for *Snail2* (brown) and *Tle3* (purple) of an HH8 embryo, arrowheads indicate *Snail2* and *Tle3* positive cells in the neural plate border. Ps- primitive streak, HN –Hensen's node, np – neural plate, eb – epiblast, hb- hypoblast, nt – neural tube, nne – non-neural ectoderm, n – notochord, aip – anterior intestinal portal. Scale bars: B' - 100µm, D'-G'' - 50µm, J-10µm.

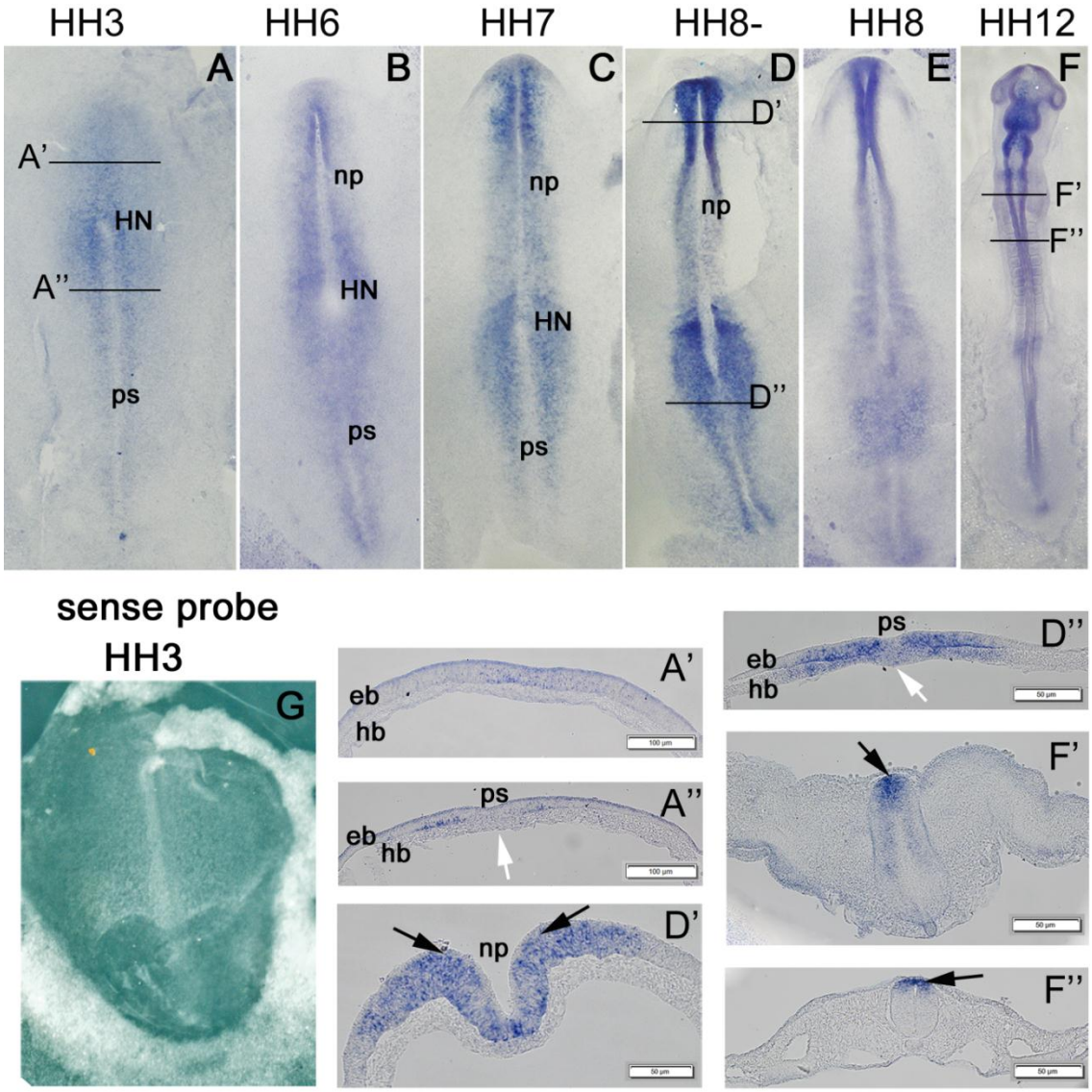


Figure 19: *Tle1* expression pattern during early chicken embryogenesis. *Tle1* is expressed in a narrow strip surrounding the primitive streak (A-D, A'', D''), the neural plate (B-E, D') and the dorsal neural tube (F'-F''). (G) HH3 embryo hybridized with the sense probe. Ps- primitive streak, HN –Hensen's node, np – neural plate, eb – epiblast, hb- hypoblast. White arrows in A'' and D'' indicate ingressing mesoderm cells, while black arrows in D'. D', F'and F'' point at the expression in the neural plate border/dorsal neural tube. Scale bars: A', A'' -100µm, D'-F''-50µm.

3.8. *Tle3* is expressed in the neural plate border

At HH4/5 *Tle3* mRNA is present in the epiblast but not in the primitive streak or ingressing mesodermal cells (Fig. 18A, B, B', white arrow). Expression at HH7 is spread throughout the epiblast and the neural plate (Fig. 18C). By HH8-, *Tle3* expression is seen in non-neural ectoderm and in the neural plate border (Fig. 18D, D', arrows). Slightly later, at HH8, we observed expression in the dorsal tip of the neural folds (Fig. 18E, arrows), non-neural ectoderm, the notochord and anterior intestinal portal (Fig. 18E, E''). By HH9, however, the very tip of the neural folds/dorsal neural tube no longer expressed *Tle3* (indicated by red arrows in Fig. 18F', F''), but *Tle3* transcripts were seen in the more ventral cells of the neural tube. At HH10, we no longer observed any *Tle3* expression in the neural tube, however, non-neural ectoderm, anterior intestinal portal and the notochord continued to express this gene (Fig. 18G, G', G''). We also performed double *in situ* hybridization with the probe for *Snail2* at HH8 (Fig. 18J). We observed co-localization of *Snail2* and *Tle3* transcripts in the neural plate border, confirming that at HH8 *Tle3* is expressed in pre-migratory neural crest cells (Appendix 2 Figure 22).

3.9. *Tle1* mRNA is present in the neural plate

During gastrulation, *Tle1* transcripts are seen in a narrow portion of the epiblast surrounding the primitive streak, and at much lower levels in the ingressing mesodermal cells (Fig. 19 A'', D'', white arrows). During neurulation, Tle1 is expressed throughout the neural plate (Fig. 19D, E, D'). By HH12 *Tle1* expression is maintained only in the dorsal-most aspect of the neural tube (arrows in F', F'').

Table 7 summarises the expression pattern of all the binding partners as well as *Prdm1* during the early stages of chick embryonic development.

3.10. All but one Prdm1 binding partners might co-operate with *Prdm1* in early stages of neural crest induction

To confirm co-expression of *Prdm1* and the putative binding partners during early stages (HH4-HH7 or HH8), double *in situ* hybridization was performed. Two probes

were used simultaneously and detected at different stages. Probes for the binding partners were labelled with DIG and the *Prdm1* probe was labelled with FITC. During the *in situ* hybridization step, the probe that took longer to develop (*Prdm1*) was first detected. This took a period of one week for the stain to be visible and clear after which the second probe was then detected using a different substrate. *Prdm1* was detected using NBT/BCIP which gives out a purple colour and the other probes were detected using INT/BCIP which gives out a brown colour. Developing/staining time for the first and second probe was not the same. Embryos did not stain the same time when hybridized with *Prdm1,* and this could have been due to the variety of eggs supplied and due to the growth of the embryo during incubation. This did not however affect the results as the embryos that were not stained within a week were allowed to stain longer before using the second substrate. INT/BCIP is soluble in alcohol unlike NBT/BCIP, which means that staining with the second probe had to be monitored carefully. Over staining of the embryos would result in no clear expression and difficulty in washing off of excess stain

The expression profile of all the binding partners shows co-expression with *Prdm1* at these early stages (HH4-HH7) (Fig. 20 and 21) except *Tle1*. *Tle1* is co-expressed with *Prdm1* at HH4-5 but at stages HH7 and HH8 these two genes appear to be present in different regions of the neural tube.

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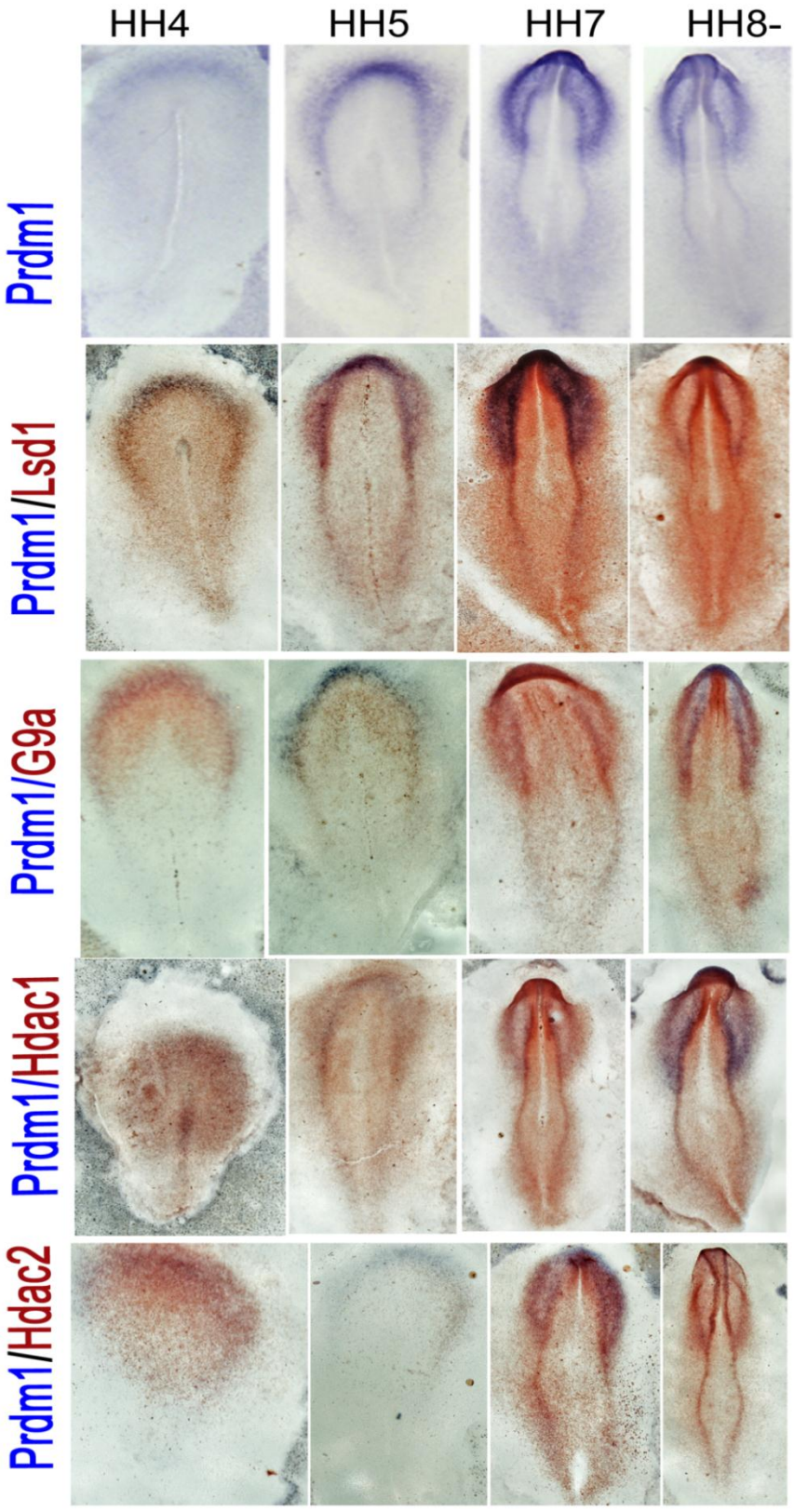


Figure 20: Double *in situ* showing co-expression of Prdm1 with G9a, Lsd1, Hdac1 and Hdac1 during early embryogenesis specifically HH4- HH7+/8-.

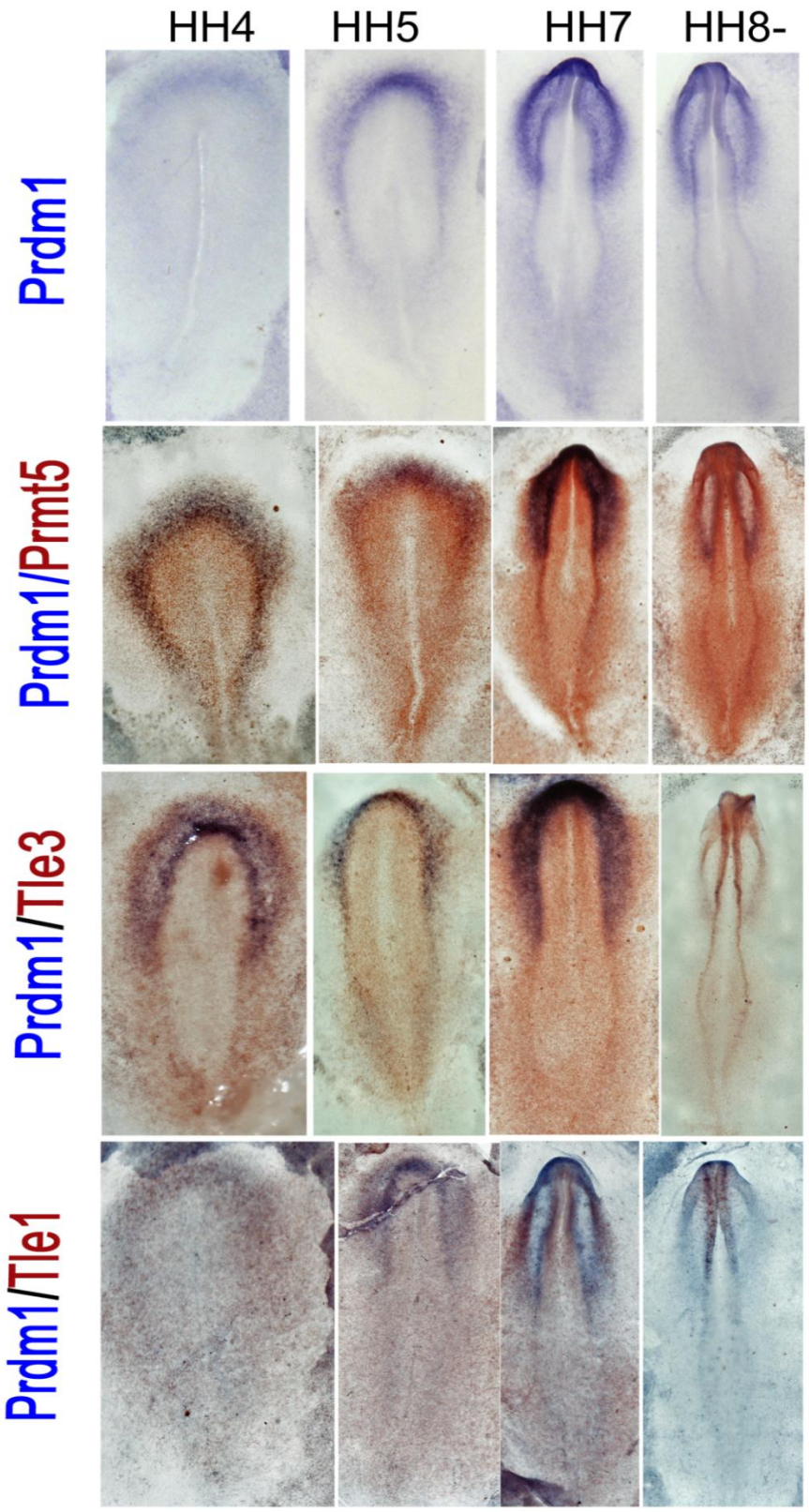


Figure 21: Double *in situ* hybridization results for Prdm1 and TLE1, TLE3 and Prmt5 individually during embryonic stages HH4- HH7/8-.

Table 7: Summary of expression of prdm1 and its binding partners (numbers in the table indicate the Hamburger-Hamilton developmental stages at which the gene is expressed in the specified anatomical location, NE – not examined, MHB- Midbrain-hindbrain region)

Anatomical location	Prdm1	G9a	LSD1	Prmt5	HDAC1	HDAC 2	TLE1	TLE3
Early embryo								
Epiblast		4-8	4-8	XII-8	4-8	4-8	4-7	4-8
Hypoblast			4-6	XII-5				
Primitive streak			4-8					
Ectoderm								
Neural plate/tube	10+ (MHB)	5-9	5-12	4-9	4-10	4-10	4-12	4-9
Optic vesicle/eyes			10-12	NE	10	10	12	NE
Neural plate border	4-8+	4-9	4-9	4-9	4-10	4-8	7-9	4-8
Neural crest				NE	10	10		NE
Pre-placodal domain	4-8	4-8	4-8	4-7	4-7	4-6		4-7
Otic placodes	9-11		10-12			9-10	NE	NE
Non-neural ectoderm	7-10+			7-8+	6-10	10		5-10
Mesoderm								
Notochord				8-9				8-10
Segmental plate		8-9	7-10	8-9	7-10		NE	NE
Somites		8-9	7-12	8-9	7-10			
Lateral plate		9	7-12	8-9	7-10		NE	
Blood islands	4-10		XII-12	XII-7	4-10	4-10		4-10
Endoderm								
Foregut	7-10+		7-10	8-9	8-10	8-10		8-9

CHAPTER4

4. Discussion

4.1. *Prdm1* is expressed in the chick neural plate border

The expression pattern of Prdm1 in the chick embryo using *in situ* hybridization has not been reported yet. IHC and qRT-PCR was used to determine expression of PRDM1 in during chick embryonic and germ-line development and germ-line. PRDM1 was detected in PGCs found in the blood and in presumptive PGCs in stage X during chicken embryonic development (Wan et al., 2014).

Our expression analysis demonstrates that *Prdm1* is present in the nascent neural crest cells from the time they are specified to just before the NC migration, suggesting a possible function during early stages of chick neural crest development. The expression of *Prdm1* transcripts in the neural plate border and the pre-placodal domain is also seen in the lamprey, zebrafish and *Xenopus* embryos (de Souza et al., 1999; Wilm and Solnica-Krezel, 2005; Nikitina et al., 2011), but not mouse embryos (Gawantka et al., 1999; Vincent et al., 2012), suggesting that the loss of *Prdm1* from the NC precursors might be a novel feature that has evolved in the mammalian or rodent lineage. Placodal cells like neural crest are ectodermal derivatives, since there is an overlap in non-neural and neural ectoderm during neurulation. It is not clearly understood when the cells of different fates separate, however gene expression marks the segregation into different cell fates (Streit, 2007). In chick embryonic development there is an overlap of neural and non-neural ectoderm, during this overlap precursors to different placodes get to intermingle with future epidermal, and NC cells.

Interestingly, we did not observe *Prdm1* expression in somites during chick embryogenesis (Fig. 12E-G, K), while somite expression of *Prdm1* is seen in all other model organisms examined to date, and it appears to play and important role in zebrafish muscle development (Gawantka et al., 1999; Hammond et al., 2009; Hernandez-Lagunas et al., 2005; Robertson et al., 2007; Roy & Ng, 2004; Wilm & Solnica-Krezel, 2005). *Prdm1* expression is switched off in the neural crest but persists in the otic placodes (Fig. 12D’’’-D’’’’). The expression of *Prdm1* in otic

placodes was also observed in lamprey (Nikitina et al., 2011). IHC studies showed an expression of PRDM1 in the chick intestine, blood vessel endothelium and the smooth muscle of the vascular wall of the lung (Wan, Rui, & Li, 2014). Our data also shows the expression of *Prdm1* in the foregut (Fig. 12 D’’’’).

Next, the expression of all known Prdm1 binding partners was examined in order to identify likely candidates that might participate in NC development. All Prdm1 binding partners that had been previously identified in adult tissues were selected, as well as some potential partners (Tle3) based on sequence similarity of these to known Prdm1 binding partners (TLE1 and TLE2, (Ren et al., 1999).

4.2. *G9a* expression pattern suggests possible roles in placode, neural tube and somite development

The expression pattern of *G9a* has not yet been reported using *in situ* hybridization in chick embryo development. Using published data on G9a interaction with Prdm1 in B-lymphocytes, we then checked its localization in the early chick embryo. *G9a* expression seems to be restricted to the areas noted in Table 7. The expression starts in the anterior epiblast and increases as the embryo grows and then goes down at later stages. We did not examine stages later than HH11; however at this stage no expression is seen in the entire embryo except the tiny region on the open neural plate. *G9a* expression is also observed in the pre-placodal region which is important in the formation of placodes. Pre-migratory neural crest cells were also observed to express *G9a* transcripts. The expression of *G9a* in the mesoderm and somites is consistent with its expression in the undifferentiated myoblast (Mei et al., 2012).

G9a is known to play a role in histone posttranslational modification especially histone lysine (K) methylation. It methylates histone 3 lysine 9 via a catalytic domain known as a SET domain. It has been shown to methylate histones by forming complexes with other proteins e.g. GLP which encodes a G9a like protein and also possesses the same substrate specificity as G9a on histones (Shinkai & Tachibana, 2011). This interaction was further confirmed in G9a or GLP knock mouse embryonic stem (ES) cells where DNA methylation was affected (Dong et

al., 2008). Furthermore this DNA methylation was independent of histone methyltransferase activity as catalytically inactive G9a partially restored the aberrant DNA methylation pattern in G9a deficient ES cells (Shinkai & Tachibana, 2011). The expression of G9a in chick embryo development also suggests that it may play a role in epigenetic modification at these early stages. Since G9a has been reported to interact with a lot of molecules, it is possible that it achieves it function via some of these interactions in chick embryo development. Most of the G9a interacting proteins are characterised as repressive chromatin proteins and multi-zinc finger molecules (Shinkai & Tachibana, 2011). G9a has been reported to interact with Prdm1 via the zinc finger domain. The co-localization of G9a and Prdm1 during early chick embryogenesis supports the possibility of this in Fig. 20; however protein interaction studies still have to be performed to prove interaction.

This work provides the first description of *in situ* expression pattern for *G9a* in chick embryo. *G9a* expression during zebrafish development is similarly seen throughout the epiblast, and later in the neural plate, neural tube and somites (Rai et al., 2010).

4.3. *Lsd1* plays a role in the specification and development of neural crest during early chick embryogenesis

Lsd1 expression has been reported in mouse embryos in the epiblast. Little or no expression was observed in the extra-embryonic tissue (Foster et al., 2010). Our data also shows expression in the epiblast and extra-embryonic tissue specifically in the blood islands. This is consistent with the hypothesis that Lsd1is important in the survival of the postimplantion embryo (Foster et al., 2010). The expression profile shows that *Lsd1* transcripts are found very early in the developing chick embryo and are persistent throughout the early stages that were examined (HH3-HH12). Hybridization with the sense probe showed no staining on the embryo which verifies the specificity of our *in situ* protocol (Fig.14 J&K). The level of expression does not seem to go down suggesting an important role of *Lsd1* in these stages. Moreover, the expression is observed in the NPB and in pre-migratory NC.

Lsd1 directly interacts with Snail1 in a complex to mediate repression of epithelial genes (Foster et al., 2010). Snail1 and Snail2 play an important role in the process of EMT during mesoderm and neural crest development. However, there have been no functional studies on the possible role of Lsd1 in neural plate border or neural crest development. *In situ* hybridization with only *Lsd1* probe at HH12 was not sufficient to prove that *Lsd1* expression persisted in the migratory NC. Double *in situ* hybridization showed co-expression of *Snail2* with *Lsd1* in the migrating NC cells. This data raises an intriguing possibility that Lsd1 may also cooperate with either Snail1 or Snail2 during NPB or NC development.

Lsd1 expression was also seen in blood islands in all the stages that were examined suggesting a role in the formation and development of blood cellular components. This is consistent with the evidence that different mass complexes of Tal/LSD1 with strong deacetylases and demethylases activity were identified during haematopoiesis. (Hu et al., 2009). To the best of our knowledge, this is the first report of spatiotemporal expression of *Lsd1* in any chick embryo.

4.4. Expression pattern of *Prmt5* suggests a role in neural crest development and possibly segmentation

Reports on *Prmt5* expression in vertebrate embryogenesis are scarce. In medaka, *Prmt* 5 appears to be ubiquitously expressed at early stages of embryogenesis (gastrulation), and continues to be widely expressed in the neural tube, eyes, somites and otic placodes by stage 18 (Chen et al., 2009). Expression in the epiblast, somites, neural tube and mesoderm observed in medaka is also consistent with the data we have collected. This suggests that Prmt5 may play a role in the development of embryonic segmentation. Our data is partly consistent with that of medaka, as *Prmt5* expression was observed throughout the epiblast and in somite (Fig. 15G’’’’’). The expression was also observed in otic placodes at later stage (HH14) though the data is not shown.

As observed in figure 15 the expression is strong in the early stages, and level of expression goes down in stages HH8 and HH9. However the level of expression

increased again at later stages (HH11 and HH14) and the data is not shown. Also expression of *Prmt5* was seen in the blood islands from HH3 –HH7 and again in stages later than HH10. *Prmt5* transcripts were expressed throughout the epiblast and in the preplacodal region, an area that gives rise to cranial placodes. The expression is then mainly observed in the neural plate and the neural plate border (Fig.15D, E). Published data showed that Ajuba and Prmt5 form a complex that is recruited to Snail repressed E-Cadherin gene (CDH1). CDH1's repression by Snail regulates EMT and thus alters cell adhesion (Karkhanis et al., 2011). Therefore it is possible that *Prmt5* expression in neural plate border suggests a role in the EMT process during neural crest development and delamination. This is the first report of expression profile of Prmt5 in chick embryonic development.

4.5. Expression pattern of Histone deacetylases 1 & 2 during the early stages of chick development is highly similar

Full length clones for Hdac1 and Hdac2 were used for *in situ* hybridization for the data published by Murko however; no expression patterns in earlier stages (earlier than HH12) were reported for both Hdac1 and Hdac2. Our primers for Hdac1 and Hdac2 were designed outside the conserved region which is highly similar to other HDACs proteins. This was done to minimise the possibility of cross-hybridization with non-target Hdac transcripts.

This is the first report of the analysis of spatial distribution of Hdac1 transcripts at early developmental stages. Murko et al. (2011) examined the levels of mRNA in early chick embryos using qPCR. They reported that Hdac1 transcripts were abundant in early embryos (HH2-HH4), then the transcript levels dropped reaching the lowest levels at HH8, and rose again by HH13-14. Our results are consistent with these findings: Hdac1 expression pattern becomes more restricted by HH8, and then becomes more wide-spread again at later stages. Here we demonstrate that *Hdac1* transcripts are expressed widely, but not ubiquitously as previously thought (Brunmeir et al., 2009; Murko et al., 2011). Expression of *Hdac1* in the migrating neural crest suggests that it might be important for neural crest development in the

chick embryo, consistently with the previous reports demonstrating its multiple important functions during zebrafish neural crest development (Pillai et al., 2004)

Hdac2 displays an expression pattern that is quite similar to that of *Hdac1*, which is probably not surprising since these two proteins are often found together in the same protein complex (Murko et al., 2010). They are both expressed in the neural plate border however *Hdac2* is not present in the migrating neural crest. Our results are consistent with the qPCR data of Murko et al. (2010), who reported that *Hdac2* mRNA in present throughout early chick embryogenesis. In mice Hdac2 appears to play an important role in neural crest specification into Schwann cell precursors and satellite glia (Jacob et al., 2014). It is not clear at present if Hdac2 plays a similar role during chick embryogenesis. Our data suggests that it is not expressed during neural crest migration at HH10-HH11. However, the transcription might be turned on at later stages, or alternatively, the protein might persist in the migrating crest after the mRNA has been degraded.

4.6. The expression pattern of the Groucho family members (TLe1 &3) during early stages of chick embryogenesis suggests different roles played by these two genes during early embryogenesis

The focus of the study was on the *Tle1* and *Tle3*. The expression pattern of these Tle genes had not been reported during the early stages of chick development. In mouse embryonic development Tle1 and Tle3 displayed similar expression pattern in some structures of an E 8.5 mouse embryo. These structures include the presomitic mesoderm subjacent to the neural plate tissue at the caudal extremity of primitive streak region (Dehni et al., 1995). Our data suggests that Tle1 and Tle3 play different roles during early development of the chick embryo based on the expression pattern. The expression of *Tle1* at HH3 is only observed in the strip surrounding the primitive streak as opposed to the expression on the primitive streak reported on previous studies (Van Hateren et al., 2005). The expression of *Tle1* is not observed in the neural plate border but is only restricted to the neural plate. Even at HH8, NC does not seem to express *Tle1* and at later stages its expression is only in the neural tube. *Tle3* however is expressed in the neural crest and this is supported

by the double *in situ* hybridization with *Snail2* at stage HH8. This data suggests that *Tle1* and *Tle3* play different roles during early development of the chick embryo.

The expression of *Tle3* in the neural crest of mouse embryos at E9.5-E10.5 is consistent with the data that showed Tle family of proteins was found to be expressed in neural crest derived tissues. They were found to be expressed in the neural crest derived ganglia of the PNS and in tissue containing neural crest cells that had emigrated from original location (Dehni, Liu, Husain, & Stifani, 1995). This data however differs significantly with our expression pattern of Tle1 which was not observed in neural crest and was only restricted to the dorsal part of the neural tube at HH12.

In situ hybridization analysis of all Tle family members was previously reported in quail (Van Hateren et al., 2005), however we found our expression patterns to differ in several respects from those reported. Firstly, we observed much more restricted early expression pattern in the case of *Tle1*. Secondly, we saw no expression of either of these genes in the somites. It is possible that the differences reflect true species-specific differenced in expression of these genes. However, the probes used by Van Hateren were designed to include the coding regions of the genes, which have high sequence similarity among the Tle paralogues, thus possibly resulting in the probes not being specific for the individual Tles. All probes used in this analysis were designed to bind within the UTR of the target mRNA.

4.7. Co-expression analysis of *Prdm1* with all the binding partners in early stages of neural crest induction

Information about co expression of the binding partners with *Prdm1* could not be obtained from single *in situ* hybridization as there was no clear expression in the neural plate border. Therefore to check for co-expression each probe (*Tle1, Tle3, Hdac1, Hdac2, Lsd1, Prmt5* and *G9a*) was mixed with *Prdm1* probe individually to perform double *in situ* hybridization. The expression profile of all the binding partners shows co-expression with *Prdm1* at these early stages (HH4-HH7) (Fig. 20 and 21). Apart from *Tle1*, all the other binding partners seem to be co–expressed

with *Prdm1* in early stages. This co-expression data highlights the possibility of prdm1 association with its binding partners during neural plate border specification as well as neural crest development. This work is at RNA level, protein studies have to be done to prove interaction. This data is new and has not been published.

It is however conceivable that Prdm1 forms part of several different protein complexes at the neural plate border. Alternatively, many of the Prdm1 binding partners have been reported to form complexes with each other. For instance, Hdac1 and Hdac2 are often found together in same complexes, e.g. Sin3, NuRD and CoREST (Brunmeir et al., 2009). Interestingly, it has been demonstrated that all of the above complexes have flexible composition, and may incorporate various histone methyltransferases such as Prmt5 (NuRD) (Guezennec et al., 2006), G9a (CoREST) (Roopra, Qazi, Schoenike, Daley, & Morrison, 2004) or Lsd1 (CoREST, NODE) (Shi et al., 2005). Additionally, different subtypes of these complexes are targeted to specific DNA sequences via association with different transcription factors or DNA binding proteins; each complex is able to form associations with a number of different TFs (Brunmeir et al., 2009).

Additionally Lsd1 is recruited to the E-cadherin and other epithelial gene promoters through interaction with Snail1 (Foster et al., 2010). It has been reported that Lsd, Hdacs and Prmt5 are involved in Snail1 dependent transcriptional repression (Foster et al., 2010; Hou et al., 2008; Peinado, Ballestar, Esteller, & Cano, 2004). It has been also shown that Tle can bind to Prdm1, which directs it to specific promoters, and then recruit Hdac1/2 to effect repression of expression via de-acetylation (Turki-Judeh & Courey, 2012). During hematopoietic differentiation LSD1 has been found to associate with HDAC1/2, CoREST and other components that are essential for its enzymatic activity and repression (Hu et al., 2009). The complexes formed by the binding partners are important in target gene repression, however it still has to be determined if all these components are in the same complex or the interaction is independent of Prdm1 or other repressors. It is however not inconceivable that multiple Prdm1-containing complexes incorporating most or all of the binding partners exist and function during neural plate border/ neural crest specification.

Further work currently under way in our laboratory will elucidate the nature of Prdm1 protein complexes in the neural plate border.

It is possible that some of the binding partners are regulated at the level of transcription. Most Hdacs including Hdac1 and 2 are enzymatically inactive and also lack the DNA binding domain, hence they are mostly found in multi-subunit complexes (Moser, Hagelkruys, & Seiser, 2014). This data suggests that any protein that recruits and associate with Hdacs can affect its enzymatic function (positively and negatively) (Sengupta & Seto, 2004). A study on MicroRNA-mediated posttranscriptional regulation in maintaining the undifferentiated state in blastoderm and primordial germ cells in chickens showed that some of the targets were down regulated (Lee et al., 2011). The somatic gene expression of HOX genes is repressed by the Prmt5 and Prdm1 complex during the formation of initial PGCs in mice (Ohitana et al, 2005). Micro RNA silencing using miRNA 181a in chicken PGCs also led to the down regulation of somatic HOXA1 (Lee et al., 2011). Studies on the regulation of the activity of Hdacs demonstrated that they can be regulated by multiple mechanisms (Sengupta & Seto, 2004). Hdac1 and 2 can be regulated by protein -protein interactions, so all the complexes formed by these Hdacs need to be discovered to better understand how Hdacs are regulated. All these findings on regulation of protein expression suggest that some of these binding partners are possibly regulated at RNA level. Whether this is the case or not, we still have to do protein interaction studies to prove direct interaction of the proposed binding partners with Prdm1 and with each other. However if these genes are regulated at the mRNA level, our data presents a significant stepping stone towards determining the roles played and complexes formed by these genes in early chick embryogenesis.

4.2. Conclusion

Our results suggest that, with the exception of *Tle1* (whose expression is restricted to the putative neural plate at earlier developmental stages), transcripts for all of the *Prdm1* binding partners examined are present in the neural plate border region from the time of gastrulation (HH4) up until the neural crest migration (HH8-9), and in the case of *Hdac1* and *Lsd1*, in the migrating neural crest (summarized in Table 2).

Since this work examined only the tissue distribution of the transcripts and not that of the proteins, further confirmation of our findings by immunohistochemistry followed by co-immunoprecipitation studies is required before any conclusions as to the nature of the Prdm1-conatining protein complexes at the neural plate border can be drawn.

4.2.1. Future work

To determine the direct interactions with binding partners, co-immunoprecipitation using antibodies against Prdm1 and each of the prospective partners to demonstrate that direct binding between these proteins occurs *in vivo* will be done. The next step would be to over-express *Prdm1* in primary neural crest culture and use antibodies against specific histone modifications to assess the change in methylation/acetylation at specific histone sites. This experiment will provide further evidence in favour of one or several of the putative mechanisms of action.

Effects of Prdm1 mis-expression on neural crest survival, proliferation and the expression of a set of neural crest specifiers and genes involved in cell cycle progression will be analysed. This will be done by exploring the effects of keeping Prdm1 expression on in the migrating neural crest cells by electroporating Prdm1-containing construct into the neural tube of the embryo at stage HH7-8. BrdU labelling will be used to assess proliferation levels and compare them to those of control embryos (electroporated with an empty construct). Cell death in the Prdm1-positive neural crest population will be assessed using TUNEL assay. Next, we will evaluate the effects of Prdm1 maintenance on the expression of several neural crest specifier genes such as Slug, Sox8 and 9, c-Myc, as well as on their downstream genes that are involved in neural crest migration, guidance and differentiation. Also long-term effects of loss and overexpression of Prdm1 on the formation of trunk neural crest derivatives will be investigated by electroporation.

CHAPTER 5

5. References

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CHAPTER 6

6. Appendices

6.1. Appendix 1: Recipes for various solutions used in this work

i. 0.5% agarose gel

0.5g of low melting agarose was mixed with 50ml of TAE/ TBE buffer, heated until it melted. After melting it was cooled down to about 55^{o}C then GR green was added. After adding GR green it was poured immediately into a gel casting tray and left to solidify.

ii. TBE or TAE

the following ingredients were added together to make 1L of 50X; Tris Base (242g), Glacial Acetic acid to make TAE (57.1) and to make TBE add Boric Acid(same amount), $Na_2EDTA.2H_2O$ (37.2) and then distilled water was added up to 1000ml. This was diluted to 1X for use in electrophoresis.

iii. Agar plates with ampicillin

To make 1L Luria broth in a flask these ingredients were added together; 2.5 g of yeast extract, 5g Bacto-Tryptone, 5g NaCl, and 7.5g agar and add distilled water to 1000ml. The flask was covered with foil and mixture was autoclaved for 20 minutes. After autoclaving it was left to cool until it could be touched with the hand without burning and then add ampicillin (500µl in 500ml).the mixture was shaken and poured immediately on plates, the plates were left on the bench to solidify. After solidifying they were incubated upside down in a 37^{o}C room or incubator.

iv. Luria Broth (LB)

To make 1L LB, 10g of Bacto-Tryptone, 5g yeast extract and 10g NaCl was added in a flask and autoclaved. After autoclaving it was

allowed to cool until it could be touched with the hand without burning and then ampicillin was added when required.

v. S.O.C medium

To make 1L of S.O, C., these ingredients were added together in 900ml of water; 20g BactoTryptone, 5g yeast extract, 2ml of 5ml NaCl, 2.5ml of 1M KCl, 10ml of 1M $MgCl_2$, 10ml of 1M $MgSO_4$ and 20ml of 1M glucose. Then the water was filled to 1l and stirred to mix the ingredients and lastly autoclaved.

vi. 4% PFA(This was done in a fume hood with gloves and a mask)

One day before making 4% PFA a 1 litre bottle with distilled water was stored in the cold room. In a 1 litre flask 600ml distilled water (not cold) was added and heated in the microwave to 65^{o}C. Then weighed 40g paraformaldehyde (PFA) in a TC fridge and then added to water. A stirrer was placed and 1-2 drops of 10N NaOH were added for each 100ml of distilled water. The solution stirred until it was clear and then 10X PBS (100ml) was added. The cold water that was stored in the cold room was added up to 900ml and when the solution was at room temperature the pH was adjusted to 7.4 using hydrochloric acid and after 100ml of water was added. 10ml aliquots of 4% PFA were then made and stored in a freezer (-20^{o}).

vii. NBT/BCIP

Nitro-blue tetrazolium / 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) was prepared by adding 9µl of NBT per 2ml of NTMT and 7µl of BCIP per 2ml of NTMT. This mixture was then filtered before it was used.

viii. PBT

To make 500ml of 10X PBS these ingredients were added together; 45g NaCl, 5.68g Na_2HPO_4, 1.2g NaH_2PO_4 and water to a volume of 450ml. the pH was adjusted to 7.4 and 50ml of water added before adding Diethylpyrocarbonate (DEPC) and autoclaving. Some of it was diluted to 1X PBS which was also DEPC treated, autoclaved and mixed with 20% Tween (500µl to 500ml) to make PBT.

ix. *NTMT* (It's important to make this on the day it has to be used)

These were the solutions prepared beforehand that were used to make NTMT:

5M NaCl (1ml), 1M Tris-HCl pH 9.5(5ml), 1M $MgCl_2$ (2.5ml), 10% Tween-20 (0.5ml) and distilled water was added to make up a volume of 50ml.

x. *MABT*

250ml of MAB (T) was made by adding 14.5g Maleic acid, 10.96g NaCl, 9.75g NaOH pellets and adding water up 200ml before adjusting pH to 7.5 and filling the bottle to 250ml. this was then autoclaved and allowed to cool before adding 20% Tween.

xi. *3% BSA*

Only the amount needed in the hybridization was made depending on the number of tubes. To make up a volume of 50ml, 1.5g of bovine albumin serum (BSA) was added to 50ml PBT.

xii. *Methanol series*

To make all the methanol series, 1X PBT DEPC treated (fresh) was mixed with different volumes of Methanol. To make 25%, 50ml methanol was added to 150ml PBT/DEPC to make 200ml. to make 50%, 100ml methanol was added to 100ml PBT/DEPC and to make 75%, 150ml methanol was added to 50ml PBT/DEPC. This was mixed in newly autoclaved bottles to prevent contamination.

xiii. *Glycerol (25%, 50% and 75%)*

This depended on the volume of glycerol required, so to make 25%, 12.5ml glycerol was added to 37.5ml PBT. To make 50%, 25ml glycerol was added to 25ml of PBT to make 50ml and as well 37.5ml glycerol was added to 12.5ml PBT to make 75% glycerol.

xiv. *Ringers solution*

To make 1 litre of Ringers, these ingredients were added; 7.2g of NaCl, 0.37 of KCl and 0.17g of $CaCl_2$. These were dissolved in

water and the pH was adjusted to 7.4. Ringers was filtered and then autoclaved before use.

xv. *Sucrose solution*

To make 100ml of 15% sucrose, 15.89 g of powder sucrose was added in water and wait for it to dissolve before filter sterilizing and storing. The number of grams was adjusted accordingly to make 30% sucrose in a volume of 100 ml.

xvi. *INT/BCIP(this has to be made on the day it has to be used)*

9µl of INT/BCIP per 2 ml of NTMT was added and the solution filtered before use.

6.2. Appendix 2: Supplementary figures

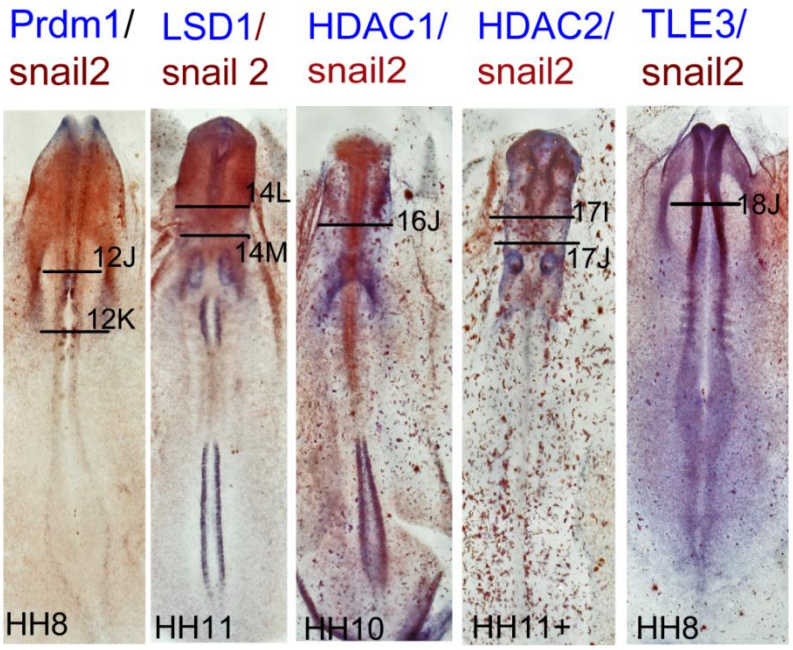


Figure 22: Double *in situ* hybridization of the respective binding partners with *Snail2*, a neural crest marker.

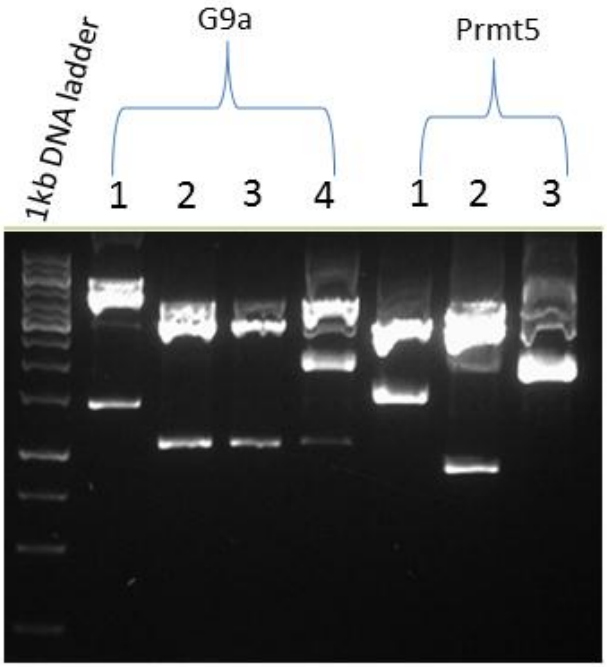


Figure 23: Representative gel showing the inserts digested out of the vector. The numbers indicate the number of different clones ran on the gel. G9a 2- 4 as well as Prmt5 2 inserts were of the correct size.