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What's RA got to do with it? Retinoic acid regulation of the neural crest in craniofacial and ocular development

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Abstract

Retinoic acid (RA), the active derivative of vitamin A (retinol), is an essential morphogen signaling molecule and major regulator of embryonic development. The dysregulation of RA levels during embryogenesis has been associated with numerous congenital anomalies, including craniofacial, auditory, and ocular defects. These anomalies result from disruptions in the cranial neural crest, a vertebrate-specific transient population of stem cells that contribute to the formation of diverse cell lineages and embryonic structures during development. In this review, we summarize our current knowledge of the RA-mediated regulation of cranial neural crest induction at the edge of the neural tube and the migration of these cells into the craniofacial region. Further, we discuss the role of RA in the regulation of cranial neural crest cells found within the frontonasal process, periocular mesenchyme, and pharyngeal arches, which eventually form the bones and connective tissues of the head and neck and contribute to structures in the anterior segment of the eye. We then review our understanding of the mechanisms underlying congenital craniofacial and ocular diseases caused by either the genetic or toxic disruption of RA signaling. Finally, we discuss the role of RA in maintaining neural crest-derived structures in post-embryonic tissues and the implications of these studies in creating new treatments for degenerative craniofacial and ocular diseases.

Keywords: vitamin A, retinoic acid signaling, cranial neural crest, craniofacial development, ocular development, auditory development

Introduction

Retinoic acid (RA) is derived from the liposoluble essential dietary nutrient vitamin A (retinol) (Cunningham & Duester, 2015; de la Cruz, Sun, Vangvanichyakorn, & Desposito, 1984; Deltour, Ang, & Duester, 1996; Rosa, Wilk, & Felsey, 1986). Many functions have been assigned to this vitamin, and studies in avian, rodent, frog, and fish models have established that the tight control of RA levels is essential for normal embryonic development, as both vitamin A deficiency and toxic exposure to retinoids lead to a complex spectrum of abnormalities (Cunningham & Duester, 2015; Das et al., 2014; de la Cruz et al., 1984; Deltour et al., 1996; Rosa et al., 1986; Vieux-Rochas et al., 2010). At the tissue level RA gradients are mediated through the differential and dynamic expression patterns of specific synthesizing (i.e., Raldh2, Raldh3, and Raldh4) and metabolizing enzymes (Cyp26a1, Cyp26b1, and Cyp26c1) (Hernandez, Putzke, Myers, Margaretha, & Moens, 2007; McCaffery, Wagner, O'Neil, Petkovich, & Drager, 1999; Molotkov, Molotkova, & Duester, 2006; Suzuki et al., 2000). RA primarily acts as a ligand for switching the transcription factors of the nuclear retinoid acid receptor (RAR$\alpha/\beta/\gamma$) and retinoic X receptor (RXR$\alpha/\beta/\gamma$) superfamily from potential repressors to transcriptional activators (Hale et al., 2006; Linville, Radtke, Waxman, Yelon, & Schilling, 2009; Nicholas Matt et al., 2005; Nicholas Matt, Ghyselinck, Pellerin, & Dupe, 2008; N. Matt, Ghyselinck,

Wendling, Chambon, & Mark, 2003; Rauch et al., 2003). Vertebrate models, from

zebrafish to mouse, and experimental tools, such as reporter transgenes,

inhibitors of RA synthesis, and selective antagonists for RARs, have been

successfully used to decipher retinoid functions during development at the

cellular and molecular levels.

Numerous examples from animal models and human diseases highlight

the importance of RA in regulating neural crest contributions to craniofacial and

ocular structures. The neural crest is a set of transient embryonic stem cells

unique to vertebrates that originate at the border of the neural plate spanning the

embryo from the diencephalon to the lumbosacral spinal cord. Neural crest cells

undergo extensive and coordinated movements away from folds of the neural

ectoderm to different regions of the embryo to give rise to a broad range of

tissues, including myofibroblasts, melanocytes, endocrine cells, neurons, glial

cells, cartilage and bone (Barembaum & Bronner-Fraser, 2005; Gammill &

Bronner-Fraser, 2003; Knecht & Bronner-Fraser, 2002; Minoux & Rijli, 2010;

Morales, Barbas, & Nieto, 2005). Cranial neural crest cells that originate from the

diencephalon and anterior mesencephalon form the frontal bone, connective

tissue of the orbit, and tendons of the extraocular muscles (Figure 1A, B, and C).

Further, a subset of these neural crest cells contribute to the sclera, iris, cornea,

ciliary body, and aqueous humor outflow pathways of the eye (Bohnsack &

Kahana, 2013; Chawla, Schley, Williams, & Bohnsack, 2016; Gong, 2014; Paul A. Trainor, 2005; P. A. Trainor & Tam, 1995). Cranial neural crest cells that delaminate from the posterior mesencephalon and rhombencephalon give rise to the cartilage and bones of the midface, middle ear, jaw and neck (Figure 1A, B, and D). Cells from the posterior mesencephalon enter into the 1st pharyngeal arch and eventually form the maxillary and zygomatic bones of the midface. Neural crest cells from the rhombencephalon, which are organized into seven separate rhombomeres, migrate into the corresponding 1st through 4th pharyngeal arches and ultimately develop into the mandible, teeth, middle ear, and hyoid (Barembaum & Bronner-Fraser, 2005; Gong, 2014; Hong & Saint-Jeannet, 2005; Minoux & Rijli, 2010; Steventon, Carona-Fontaine, & Mayor, 2005; Paul A. Trainor, 2005; Williams & Bohnsack, 2015). Multiple complex pathways and factors, including RA, regulate neural crest induction, migration, survival and differentiation (Barembaum & Bronner-Fraser, 2005; Gammill & Bronner-Fraser, 2003; Gong, 2014; Hong & Saint-Jeannet, 2005; Minoux & Rijli, 2010; Morales et al., 2005; Steventon et al., 2005; Paul A. Trainor, 2005). Here, we review the main functions of retinoid signaling in regulating the neural crest in the context of craniofacial and ocular development.

RA and Cranial Neural Crest Induction

Neural crest cell specification occurs between the neural plate border and the non-neural ectoderm during and immediately after gastrulation. As the neural folds come together at the dorsal midline, the neural crest cells that now lie between the overlying epidermis and the neural tube, undergo an epithelial to mesenchymal transition and delaminate from the neuroepithelium (Barembaum & Bronner-Fraser, 2005; Grenier, Teillet, Grifone, Kelly, & Duprez, 2009; Knecht & Bronner-Fraser, 2002). Studies in rodents, birds, frogs, and zebrafish have shown that the neural plate border is primed by BMP signaling gradients, but a second signal, including RA and certain members of the FGF or Wnt family proteins, are required for the induction of *Gbx2*, one of the earliest neural crest markers (Barembaum & Bronner-Fraser, 2005; Knecht & Bronner-Fraser, 2002; LaBonne & Bronner-Fraser, 1998; Li, Lao, & Joyner, 2005; Mayor, Morgan, & Sargent, 1995; Streit & D., 1999; von Bubnoff, Schmidt, & Kimelman, 1996; Wawersik, Evola, & Whitman, 2005).

Specifically, RA is important in the anteroposterior patterning of the neural tube and the demarcation of the anterior and posterior neural plates (Papalopulu & Kitner, 1996; Villanueva, Glavic, Ruiz, & Mayor, 2002; Wu, Yang, & Klein, 2005). In frogs, RA suppresses the expression of *ag1* at the anterior border and induces the expression of *snai2,* an important regulator of the epithelial-mesenchymal transition in premigratory neural crest cells at the posterior border

(Villanueva et al., 2002). To define the telencephalon, which does not give rise to neural crest cells, endogenous RA levels are tightly regulated by the localized expression of *Cyp26a1* and *Cyp26c1* (Alexandre et al., 1996; Dubey, Rose, Jones, & Saint-Jeannet, 208; Yu et al., 2016). At the posterior neural plate, *Cyp26a1* expression is inhibited in a RA-dependent fashion via FGF and Wnt signaling resulting in increased RA levels (Kudoh, Wilson, & Dawid, 2002). Exogenous treatment with RA in zebrafish, frogs, and rats during and immediately after gastrulation disrupts the anteroposterior patterning and alters cranial neural crest induction from the diencephalon, mesencephalon, and rhombencephalon (Alexandre et al., 1996; Chawla et al., 2016; Gitton et al., 2010; Y. M. Lee et al., 1995). In addition, in double *Cyp26a1 -/-* and *Cyp26c1 -/-* knockout mice, the posteriorization of the anterior neural tube results in the absence of neural crest cells from the diencephalon and mesenecephalon (Uehara et al., 2007). In these animal studies, increased RA leads to indistinct populations of cranial neural crest cells that are unable to migrate properly from the edge of the neural tube. Therefore, the tight control of RA levels within the developing brain is critical for neural crest induction and the initiation of cell migration into the craniofacial region.

RA Regulation of Cranial Neural Crest Cells in the Periocular Mesenchyme and Frontonasal Process

Following induction, cranial neural crest cells undergo epithelial-mesenchymal transition and migrate along defined pathways from the neural tube into the craniofacial region and pharyngeal arches (Paul A. Trainor, 2005). In zebrafish, time-lapse imaging demonstrated that cranial neural crest cells from the diencephalon follow a migratory stream dorsal to the developing eye while cells originating from the anterior mesencephalon track ventral to the eye; these cells populate the periocular mesenchyme and then converge in the frontonasal process, which is ventral to the prosencephalon (Figure 1A) (Bohnsack & Kahana, 2013; Bohnsack, Kasprick, Kish, Goldman, & Kahana, 2012; Chawla et al., 2016).

During neural crest cell migration, RA activity is highest within the prosencephalon and developing eye (Chawla et al., 2016; Niederreither, Vermot, Fraulob, Chambon, & Dolle, 2002). In zebrafish, the cranial neural crest cells via RARα RARγ migrate toward the frontonasal process and developing eye, areas of high RA activity, but circumvent areas of low RA activity. Exogenous RA or treatment with a RARα or RARγ agonists at the initial stage of cell migration decreases survival and inhibits the migration of the remaining neural crest into the periocular mesenchyme and frontonasal process. This inhibition results in

underdeveloped corneas and irides and abnormal midline facial structures. This effect of exogenous RA is in part due to the RARα-mediated downregulation of the homeobox transcription factor, *pitx2* in neural crest cells migrating from the anterior mesencephalon. In zebrafish, knockdown of *pitx2* inhibits the ventral but not the dorsal stream of cranial neural crest cells (Chawla et al., 2016). As a result, there are fewer neural crest cells in the periocular mesenchyme and frontonasal process in *pitx2* knockdown fish. This finding gives insight into the midface hypoplasia and anterior segment dysgenesis observed in humans with Axenfeld-Rieger syndrome (OMIM:180500) due to *PITX2* mutations (Dressler et al., 2010; Hjalt & Semina, 2005; Idrees et al., 2006; Tumer & Bach-Holm, 2009).

The frontonasal process gives rise to midline facial structures, including part of the maxillary bone (philtrum region), the frontal bone and the avian beak. Through RARα□and RARγ, RA regulates the frontonasal skeleton. In mice, the combined knockout of RARα□and RARγ causes midfacial clefts (absence of and incomplete midline fusion of portions of the frontal, maxillary, and sphenoid bones) due to the mislocalization of apoptosis in midline neural crest cells (Dupe & Pellerin, 2009). Further, RA is required for osteoblast differentiation from neural crest cells in the presumptive frontal bone. However, exogenous RA alters the balance between osteoblast and osteoclast activity in bone remodeling resulting in a thickened calvarium (Ferguson, Devarajan, & Atit, 2018). In chick, RA is

concentrated in the frontonasal process and induces FGF and SHH signaling in the surrounding ectoderm to initiate beak formation (Schneider & Helms, 2003; Schneider, Hu, Rubenstein, Maden, & Helms, 2001). Again, the tight control of RA levels is critical, as increased RA in the frontonasal process leads to the doubling of midline structures, including the beak (S. H. Lee, Fu, Hui, & Richman, 2001).

Few studies have investigated the molecular regulation of neural crest migration and the differentiation of these cells into structures in the anterior segment of the eye. In zebrafish, neural crest cells migrate in two pathways: 1) between the optic cup and surface ectoderm and 2) through the ocular fissure. These neural crest cells coalesce around the lens and give rise to the corneal stroma, the iris stroma, and the aqueous outflow channels (Eason, Williams, Chawla, Apsey, & Bohnsack, 2017). In humans there are 3 separate migratory waves of neural crest cells that sequentially contribute first to the cornea, then to the iris, and finally to the aqueous outflow tract. However, in the chick, there are 2 waves of migrating cells, and in zebrafish and mice, there is 1 continuous influx of neural crest cells into the developing eye. Despite these species differences, the general sequence of anterior segment formation remains consistent (Eason et al., 2017; Gage, Rhoades, Prucka, & Hjalt, 2005; E. Hay, 1979; E. D. Hay & Revel, 1969; Pei & Rhodin, 1970).

In humans, birds, mice, and zebrafish, the genetic or pharmacologic disruption of RA signaling causes corneal, iris and aqueous outflow tract defects which can lead to blindness (Aldahmesh, Khan, Hijazi, & Alkuraya, 2013; Bohnsack et al., 2012; Casey et al., 2011; Fares-Taie et al., 2013; Golzio et al., 2007; Pasutto et al., 2007; Roos et al., 2014; Srour et al., 2013). The local source of RA is the eye itself, as RA-synthesizing enzymes are expressed in the dorsal and ventral retina and are separated by cranial and caudal *Cyp26c1* expression (Bohnsack et al., 2012; Cvekl & Wang, 2009; Gregg Duester, 2009; Molotkov et al., 2006; Williams & Bohnsack, 2015). This specific spatial gene expression pattern creates dorsal and ventral RA gradients centered in and around the eye that through RARβ☐and RARγ, directly target the periocular mesenchyme but not the optic cup (Figure 1A0 (Nicholas Matt et al., 2005; Nicholas Matt et al., 2008; N. Matt et al., 2003; Williams & Bohnsack, 2015).☐In mice and zebrafish, RA increases Pitx2 expression within the periocular mesenchyme, unlike in the early ventral neural crest. This increased Pitx2 expression in turn regulates the migration and differentiation of ocular neural crest cells through several downstream pathways, including *dlx2* and the canonical Wnt signaling system (Bohnsack et al., 2012; Gage, Qian, Wu, & Rosenberg, 2008; Kumar & Duester, 2010; Zacharias & Gage, 2010).

Taken together, the cranial neural crest that originates from the diencephalon and anterior mesencephalon requires RA at numerous steps during the migration and differentiation of these cells into structures in the midline of the face and anterior segment of the eye. Alteration in RA levels, either increased or decreased, results in congenital malformations such as cleft lip, cleft palate, and anterior segment dysgenesis of the eye.

RA and Pharyngeal Arch Development

The pharyngeal arches are a set of six outpouchings that develop on both sides of the pharynx and give rise to numerous head and neck structures including the maxilla, mandible, teeth, middle ear bones, striated facial muscles and mucous membranes lining the pharynx (Graham, 2003; Graham & Smith, 2001; Mark, Ghyselinck, & Chambon, 2004). Each arch comprises a mesodermal core surrounded by endoderm on the pharynx side and epithelium on the outside. Then, neural crest cells from the posterior mesencephalon and consecutive rhombomeres of the rhombencephalon migrate into the specific arches and interact with the three surrounding germ layers (Figure 2) (Graham, 2003; Graham & Smith, 2001; Mark et al., 2004).

The neural crest cells within the first pharyngeal arch give rise to the maxillary and mandibular bones of the face. In mice and zebrafish, maxillary

bone formation is dependent on the epithelial-expressed FGF8-mediated induction of *Dlx1* and *Dlx2* expression in the neural crest (Chai & Maxson, 2006; Minoux & Rijli, 2010; Park et al., 2004; Shigetani et al., 2002). In contrast, the mandible is regulated by the endothelin-1 (Et1)-induced expression of *Dlx5* and *Dlx6* (Charite et al., 2001; Ozeki, Kurihara, Tonami, Watatani, & Kurihara, 2004; Ruest, Xiang, Lim, Levi, & Clouthier, 2004). In zebrafish, exogenous treatment with RA after neural crest induction and delamination suppresses mandible formation and, to a lesser extent, maxilla development. Further, in mice, a pulse of RA during somitogenesis altered the mandible into a maxilla-like structure. In these studies, RA decreased both *Fgf8* and *Et1* expression within their restricted domains in the pharyngeal arch. However, RA had a greater effect on suppressing the expression of *Dlx5* and *Dlx6* compared to that of *Dlx1* and *Dlx2,* which may account for the alteration in neural crest cell fate (Abe, Maeda, & Wakisaka, 2008; Ellies, Langille, Martin, Akimenko, & Ekker, 1997; Vieux-Rochas et al., 2007)

The patterning of the neural crest cells within the second through the fourth pharyngeal arches is determined by the expression of Hox (homeobox) transcription factors from premigratory cells in the rhombomeres (Figure 2) (Hunt, Gulisano, et al., 1991; Hunt, Whiting, et al., 1991; Lumsden & Krumlauf, 1996; P. A. Trainor & Krumlauf, 2000). For example, across mice, frogs, and fish, *Hoxa2*

is expressed up to the border of the first and second rhombomeres and is critical in conferring the identity of the second pharyngeal arch. The knockout or downregulation of *Hoxa2* results in the transformation of the second arch into a first arch, leading to the loss of the hyoid bone and the redundancy of the first arch-derived maxillary and mandibular structures (Baltzinger, Ori, Pasqualetti, Nardi, & Rijli, 2005; Gendon-Maguire, Mallo, Zhang, & Gridley, 1993; Hunter & Prince, 2002; Rijli et al., 1993). Similarly, *Hoxa3* and *Hoxa4* (and their paralogs) are important in conferring the identity of the third and fourth pharyngeal arches (Chisaka & Capecchi, 1991; Condie & Capecchi, 1994; Manley & Capecchi, 1995, 1997). However, in the more posterior arches, it is the specific combination of *Hox* genes that ultimately directs the development of the neural crest from each pharyngeal arch into their respective skeletal structures (Minoux, Antonarakis, Kmita, Duboule, & Rijli, 2009).

RA is a key regulator of Hox genes within the rhombomeres. The expression of *Cyp26a1, Cyp26b1,* and *Cyp26c1* spatially and temporally patterns the rhombencephalon to create a RA gradient with the lowest levels expressed anteriorly and the highest levels expressed posteriorly (Figures 2) (Dubey et al., 208; Mark et al., 2004). In frogs, the differential sensitivity of the various Hox-2 family genes to RA establishes the anterior to posterior identity of the neural crest cells and directs their migration from the rhombomeres into the

corresponding pharyngeal arches (Dekker et al., 1992). Further, in zebrafish, chick and mice, exogenous retinoic acid treatment during gastrulation and early somitogenesis alters the Hox code, transforming rhombomeres 2 and 3 into a more posterior identity. This transformation results in the enlargement of the neural crest-derived hyoid cartilage in zebrafish and the conversion of the trigeminal nerve into a facial nerve in mice (Alexandre et al., 1996; Mark et al., 1995; Marshall et al., 1992; Plant, MacDonald, Grad, Ritchie, & Richman, 2000).

Following neural crest migration from the rhombencephalon into the pharyngeal arches, RA primarily targets the endoderm and ectoderm and, through interactions with the adjacent neural crest, has subsequent effects on these cells (Mark et al., 2004; N. Matt et al., 2003; Muller et al., 1996; Veitch, Begbie, Schilling, Smith, & Graham, 1999; Wending, Dennefeld, Chambon, & Mark, 2000). The RA gradient established within the rhombencephalon is maintained within the pharyngeal arches, with the lowest concentrations of RA found more anteriorly. Thus, the second pharyngeal arch is only mildly sensitive to RA deficiency, as this structure is absent in *Raldh2* knockout mice, but not in *raldh2* null zebrafish or in *RARα□RARγ□*null mice (Begemann, Schilling, Rauch, Geisler, & Ingham, 2001; Niederreither, Subbarayan, Dolle, & Chambon, 1999; Wendling, Ghyselinck, Chambon, & Mark, 2001). On the other hand, exposure to isotretinoin in humans or exogenous RA in mice during gastrulation results in the

fusion and hypoplasia of the first and second arches, which is mediated by RARβ–RXR heterodimers (Lammer et al., 1985; N. Matt et al., 2003). The third through the sixth pharyngeal arches are highly sensitive to RA deficiency in humans, rodents, birds, frogs, and zebrafish. Vitamin A deficiency in chick or the genetic knockout of *RARα*, *RARβ*, *RARα*, *RARγ*, or *Raldh2* in mice and zebrafish causes hypoplasia of the four posterior pharyngeal arches (Begemann et al., 2001; Dupe, Ghyselinck, Wendling, Chambon, & Mark, 1999; Maden, Gale, Kostetskii, & Zile, 1996; Mendelsohn et al., 1994; Niederreither et al., 1999; Niederreither et al., 2003; Vermot, Niederreither, Garnier, Chambon, & Dolle, 2003; Wendling et al., 2001; White, Highland, Kaiser, & Clagett-Dame, 2000). In humans, the effects of vitamin A deficiency on the posterior pharyngeal arches most commonly manifest as cardiac anomalies, such as aortic arch, aorticopulmonary septal and ventricular septal defects (Mark et al., 2004).

RA deficiency in humans and animals is also associated with congenital hearing loss due to malformations of the bones of the middle ear (Emmett & West, 2014; Romand, 2003). In rodents, birds, frogs, and fish, abnormal rhombencephalon patterning due to alterations in RA signaling also affects the malleus and incus, which originate from the mandibular portion of the 1st arch, and the stapes, which arises from the 2nd arch (Kil et al., 2005; Maden et al., 1996; Niederreither et al., 1999; Pittlik, Domingues, Meyer, & Begemann, 2008;

White et al., 2000). However, in mice RA-induced transformation of the 2nd arch identity did not cause duplication of the malleus and incus and loss of the stapes. Instead, exogenous RA treatment during early somitogenesis resulted in absence of the malleus and incus and malformation of the stapes. These studies showed that independent of Hox gene-induction, RA directly regulates migration of neural crest cells destined for the middle ear (Mallo, 1997).

Taken together, RA is critical for pharyngeal arch development and subsequent formation of the head and neck. RA is required at multiple steps within the rhombomeres of the brain and within the individual pharyngeal arches. Disruption of RA in humans and animal models can result in craniofacial malformations and hearing loss.

Congenital Craniofacial and Ocular Diseases Resulting from the Disruption of RA Signaling

Similar to animal studies, both decreases and increases in RA signaling are associated with congenital craniofacial, ear, and ocular anomalies in humans (Gitton et al., 2010; Lampert et al., 2003; Sandell et al., 2007). (Akhtar et al., 2013; Jans et al., 2015; West, 2003; Zile, 1998). The embryo is dependent on maternal sources of vitamin A, which are in the form of retinol, retinyl esters and β-carotene (Spiegler, Youn-Kyung, Wassef, Shete, & Quadro, 2012). Although uncommon in developed countries, fetal vitamin A deficiency is typically

associated with cleft lip, cleft palate and microphthalmia (small disorganized eye). As detailed throughout this review, animal models including rodents, birds, frogs, and zebrafish have yielded important insight into the pathogenesis of these findings. For example, RAR knockout mice demonstrate that decreased RA signaling impairs midline fusion of facial bones arising from the frontonasal process and the 1st pharyngeal arch resulting in cleft lip and palate (Begemann et al., 2001; Bohnsack et al., 2012; Bohnsack, Lai, Dolle, & Hischi, 2004; Lohnes et al., 1994; Maden et al., 1996; N. Matt et al., 2003; Mendelsohn et al., 1994; Niederreither et al., 2002; Niederreither et al., 2003; See & Clagett-Dame, 2009; Wai-Man See & Clagett-Dame, 2009; White et al., 2000)

Decreased RA signaling is also hypothesized to contribute to the craniofacial and ocular defects caused by prenatal alcohol exposure. The most severe form, fetal alcohol syndrome, is characterized by a thin vermillion (thin upper lip), shortened palpebral fissures (decreased eyelid opening), smooth philtrum (no groove between the nose and upper lid), low nasal bridge, micrognathia (small jaw), microphthalmia, and in severe cases, cleft lip and cleft palate (Foroud et al., 2012; Hoyme et al., 2005; Klingenberg et al., 2010; Popova et al., 2016; Riley, Infante, & Warren, 2011). The variability of phenotype in this congenital disease is due to differences in the timing and amount of alcohol exposure during embryogenesis. Bird, rodent, frog, and zebrafish models of fetal

alcohol syndrome utilize different exposure times and at various stages to understand the mechanisms underlying tissue damage (Joya, Garcia-Algar, Vall, & Pujades, 2014; Kiecker, 2016; Petrelli, Bendelac, Hicks, & Fainsod, 2019; Riley et al., 2011; Sulik, Johnson, & Webb, 1981; Sulik, Lauder, & Dehart, 1984; Zhang et al., 2014). The original hypothesis proposed that ethanol is a competitive inhibitor of the aldehyde dehydrogenase enzymes that are also important in the conversion of retinol to RA (Deltour et al., 1996; G. Duester, 1991). Consistent with this idea, exogenous RA partially rescues the effects of alcohol in zebrafish embryos (Marrs et al., 2010; Muralidharan, Sarmah, & Marrs, 2015). In addition, similar to the disruption of RA signaling, ethanol exposure in chick decreases neural crest cell migration from the rhombencephalon into the pharyngeal arches, resulting in mandibular and maxillary defects (Kiecker, 2016). However, differences in molecular pathways and phenotypes suggest that ethanol may increase or decrease RA in a tissue-specific manner (Reijntijes, Blentic, Gale, & Maden, 2005). In addition, ethanol has been shown to have a number of RA-independent effects, including interfering with SHH, Wnt, and FGF signaling pathways and increasing oxidative stress which may account for the differences in phenotypes between vitamin A deficiency and fetal alcohol syndrome (Ahlgren, Thakur, & Bronner-Fraser, 2002; Aoto, Shikata, Higashiyama, Shiota, & Motoyama, 2008; Ashwell & Zhang, 1996; S. Y. Chen & Sulik, 1996; Chrisman et

al., 2004; Chu, Tong, & de la Monte, 2007; Garic, Flentke, Amberger, Hernandez, & Smith, 2011; Heaton, Paiva, Mayer, & Miller, 2002; Henderson, Devi, Perez, & Schenker, 1995; Koch, Galeotti, Bartoli, & Boveris, 1991; Ornoy & Ergaz, 2010; Videla, Fraga, Koch, & Boveris, 1983; Yelin, Kot, Yelin, & Fainsod, 2007).

Excess maternal vitamin A intake or, more commonly, exposure to RA analogs, such as isotretinoin, cause craniofacial anomalies, including microtia (small ears), hypertelorism (wide spaced eyes), cleft palate, cleft lip, micrognathia (small jaw), and midface hypoplasia (Maden, 2001; Rosa et al., 1986; See & Clagett-Dame, 2009; Zile, 1998). Animal models of RA embryopathy, as described throughout this review, have pinpointed specific time frames in rodents, birds, frogs, and zebrafish, during which different cranial neural crest populations are sensitive to increased RA exposure. For example, increased RA during gastrulation and early somitogenesis decreases neural crest cell migration from the diencephalon and mesencephalon. Consequently, the decrease in neural crest cells in the frontonasal process and 1st pharyngeal arch accounts for the midface hypoplasia and micrognathia. (Alexandre et al., 1996; Chawla et al., 2016; Gitton et al., 2010; Y. M. Lee et al., 1995; Uehara et al., 2007). Taken together, these studies demonstrate that short pulses of RA throughout gastrulation and somitogenesis have deleterious effects on the

pharyngeal arches and frontonasal process, resulting in irreversible jaw, middle

ear, and midface abnormalities that recapitulate the human findings (Abe et al.,

2008; Alexandre et al., 1996; Chawla et al., 2016; Dekker et al., 1992; Ellies et

al., 1997; Gitton et al., 2010; Hart, McCue, Ragland, Winn, & Unger, 1990; S. H.

Lee et al., 2001; Y. M. Lee et al., 1995; Mallo, 1997; Mark et al., 1995; Marshall

et al., 1992; Plant et al., 2000; Vieux-Rochas et al., 2007).

In addition to the pharmacologic manipulation of RA levels, genetic

alterations in RA signaling are important causes of congenital craniofacial and

ocular defects (Adams & J., 1995; de la Cruz et al., 1984; Lammer et al., 1985;

Maden, 2001; Rosa et al., 1986). Mutations in genes that are important for

regulating RA synthesis (*ALDH1A3*, OMIM:600463; *CHD7,* OMIM:608892),

degradation (*CYP26B1,* OMIM:605207*),* transport (*STRA6*; OMIM:610745), and

signaling (*RARβ*☐☐☐☐☐☐☐☐☐☐☐☐) have all been identified in congenital

diseases (Aldahmesh et al., 2013; Casey et al., 2011; Chassaing et al., 2013;

Fares-Taie et al., 2013; Golzio et al., 2007; Laue et al., 2011; Micucci et al.,

2014; Pagon, Graham Jr., Zonana, & Young, 1981; Pasutto et al., 2007; Roos et

al., 2014; Srour et al., 2013; Yahyavi et al., 2013; Yao et al., 2018) Further,

known downstream targets of RA within neural crest cells, including *RAI1*

(OMIM:607642), *FGFR1* (OMIM:136350), *COL2A1* (OMIM:120140), *TBX1*

(OMIM:602054), and *PITX2* (OMIM:601542), have also been associated with

craniofacial and ocular anomalies (Bellus et al., 1996; Bohnsack et al., 2012; L. Chen et al., 2016; Dressler et al., 2010; Gage et al., 2008; Goodship, Cross, LiLing, & Wren, 1998; Greenberg et al., 1996; Harris et al., 2004; Hjalt & Semina, 2005; Janesick, Shiotsugu, Taketani, & Blumberg, 2012; Kumar & Duester, 2010; Y.-W. Lee et al., 2012; Okubo et al., 2011; Patil & Bartley, 1984; Smith et al., 1986; Stickler, Hughes, & Houchin, 2001; Zacharias & Gage, 2010). This list will no doubt continue to expand as additional genetic testing associates other genes important in the RA signaling pathway with rare congenital craniofacial and ocular diseases.

RA in Maintaining Neural Crest-Derived Structures in the Adult

Studying the molecular mechanisms that underlie development is not only important for understanding the basis of congenital anomalies but also gives insight into the pathogenesis of the degenerative diseases that affect these tissues. Notably, RA is paramount in postnatal tissues. Vitamin A deficiency, reflecting poor diet, malabsorption, or liver disease, is characterized by impaired immunity and hematopoiesis and causes skin rashes and ocular problems, e.g., nyctalopia (night blindness), xerophthalmia (decreased tear production), and keratomalacia (corneal thinning and opacification) (Cañete, Cano, Muñoz-Chápuli, & Carmona, 2017; Lai, Ng, & Srinivasan, 2014; Parafita-Fernández et

al., 2015). In bone, RA maintains mineral density, as both increased and decreased levels are associated with increased risk of fractures in elderly individuals (Maggio et al., 2006; Melhus et al., 1998; Opotowsky, Bilezikian, & NIf-u, 2004; Promislow, Goodman-Gruen, Slymen, & Barrett-Connor, 2002). RA regulates the balance between osteoblast and osteoclast differentiation and activity; however, the specific effect is dependent on the bone type and location(Lind et al., 2017).

In a recent study, we showed that in neural crest-derived craniofacial and ocular tissues, there is a continued need for RA in maintaining the function and integrity of these structures (Chawla, Swain, Williams, & Bohnsack, 2018). In adult zebrafish, the neural crest-derived craniofacial bones, including the jaw and frontal bone, are highly sensitive to exogenous RA and the inhibition of endogenous RA synthesis. Within 5 days of altering RA levels, morphogenic changes due to bone remodeling and apoptosis in the jaw and frontal bone were observed. As expected, RA deficiency caused blindness due to the deprivation of substrate for phototransduction in the adult zebrafish retina. However, both increased and decreased RA had additional effects on neural crest-derived anterior segment structures, including the cornea and iridocorneal angle, which resulted in decreased aqueous outflow from the eyes (Chawla et al., 2018). This study represents the first evidence for the tight control of RA levels to maintain

the normal structure and function of the anterior segment of the adult eye. Additional studies are required to determine whether RA plays a role in degenerative diseases affecting the anterior segment of the eye, such as primary open angle glaucoma or keratoconus. Moreover, it would also be interesting to determine whether these effects are also observed in mammals. Nevertheless, RA plays a continued role in maintaining neural crest-derived structures in post-embryonic tissues. Further, the deleterious effects of alterations in RA levels have implications in the therapeutic use of retinoids in humans and the environmental toxic exposures to animals.

Future Directions and Challenges

RA is critical for craniofacial and ocular development. As a nutrient-based signaling factor, RA levels can be increased or decreased which can unfortunately result in severe birth defects. Vitamin A deficiency continues to be a serious global health issue, especially in regions with limited access to fresh fruits and vegetables and supplemental vitamins. Further, the therapeutic use of retinoids and excess vitamin A is also a public health concern. Numerous studies have worked to characterize both the essential and detrimental roles of RA during embryogenesis. Within craniofacial and ocular development, RA regulates almost all steps of neural crest induction, migration, proliferation, survival and

differentiation. Thus, pharmacologic or genetic alterations in RA signaling cause congenital craniofacial, ear, or eye defects that result in disfiguration, deafness, and blindness. Improving our understanding of the mechanisms that underlie the RA-mediated regulation of neural crest development will lead to increased knowledge of the pathogenesis of these birth defects and provide breakthroughs in treatments for affected individuals.

In addition, studying the signaling pathways important for the migration and differentiation of embryonic neural crest cells may lead to new treatments for degenerative diseases that affect craniofacial and ocular neural crest-derived structures. The signals that are important during embryogenesis, such as RA, have recently been shown to play roles in adult tissues. Modifying or enhancing these pathways may yield new therapeutic targets for adult diseases. In addition, one of the greatest therapeutic challenges within the anterior segment of the eye is the lack of a known neural crest-derived stem cell population that can regenerate cells for cornea, iris, or aqueous outflow tracts. Determining embryonic markers and signaling pathways within embryonic ocular neural crest cells will lead to the identification of an adult neural crest stem cell population that could potentially restore vision.

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Figure Legends

Figure 1. Neural crest derivatives in the craniofacial region. A) The neural crest is a transient population of embryonic stem cells that delaminate from the edge of the neural tube spanning from the diencephalon (Di) to the lumbosacral spinal cord (SpC). Neural crest cells that originate from the diencephalon and anterior mesencephalon (AM) migrate dorsal and ventral to the eye to populate the periocular mesenchyme (POM) and frontonasal process (FNP). These cells migrate toward regions of high RA levels within the telencephalon (Te), periocular mesenchyme and frontonasal process. Neural crest cells from the posterior mesencephalon (PM) migrate into the 1st pharyngeal arch. Neural crest cells from the rhombencephalon, which are patterned by a RA gradient, migrate into the 1st through 4th pharyngeal arches in an anterior (AR) to posterior (PR) pattern. **B)** The cranial neural crest is important in the development of the craniofacial skeleton. Neural crest cells in the frontonasal process give rise to the frontal bone (Fr), nasal bone (Na), and philtrum (Ph) in the midline of the face. The anterior portion of the 1st pharyngeal arch forms the maxillary (Mx) and zygomatic (Zy) bones while the posterior aspect gives rise to the mandible (Mn), The 3rd and 4th pharyngeal arches both contribute to the hyoid (Ny) bone in the neck. **C)** Neural crest cells, which are derived from the anterior mesenchyme and

diencephalon, populate the periocular mesenchyme and migrate into the anterior segment of the eye. Ocular structures derived from the neural crest include the corneal stroma (CS), corneal endothelium (CEn), trabecular meshwork (TM), sclera (Scl), iris stroma (IS), uveal melanocytes (Me), ciliary body muscle (CBM), and the tendons of the extraocular muscles (Tn). The corneal epithelium (CEp) and lens are derived from surface ectoderm while the ciliary body epithelium (CBE), retina (Ret), and retinal pigmented epithelium (RPE) arise from neuroepithelium. Schlemm's canal (SC) and extraocular muscles (EOM) originate from mesoderm. **D)** Neural crest cells are also important in middle ear development. Vibrations are transmitted from the external ear, which consists of the pinna, auditory canal, and the tympanic membrane (Tym). The tympanic membrane is attached to the malleus in the middle ear. Both the malleus and the incus originate from the 1st pharyngeal arch. The vibration is then transmitted to the stapes, which arises form the 2nd pharyngeal arch. The eustachian tube (Eus) connects the middle ear to the nasopharynx.

Figure 2. RA signaling and pharyngeal arch formation. A) Expression of RA synthesis (Raldh2) and degradation (Cyp26) enzymes within the rhombencephalon creates a gradient in which RA levels are lowest anteriorly and

highest posteriorly. The maxillary versus mandibular fate of neural crest cells within the 1st pharyngeal arch is dictated by *Dlx1/2* and *Dlx5/6*, respectively. The RA gradient along with spatial expression of RARs regulates the Hox genes within the 2nd through 7th rhombomeres (R2-R7). The specific combination of Hox gene expression patterns the premigratory neural crest cells with the rhombencephalon and confers the identity of the 2nd through 4th pharyngeal arches that is necessary for subsequent differentiation into their respective skeletal, cartilaginous, and nerve elements.
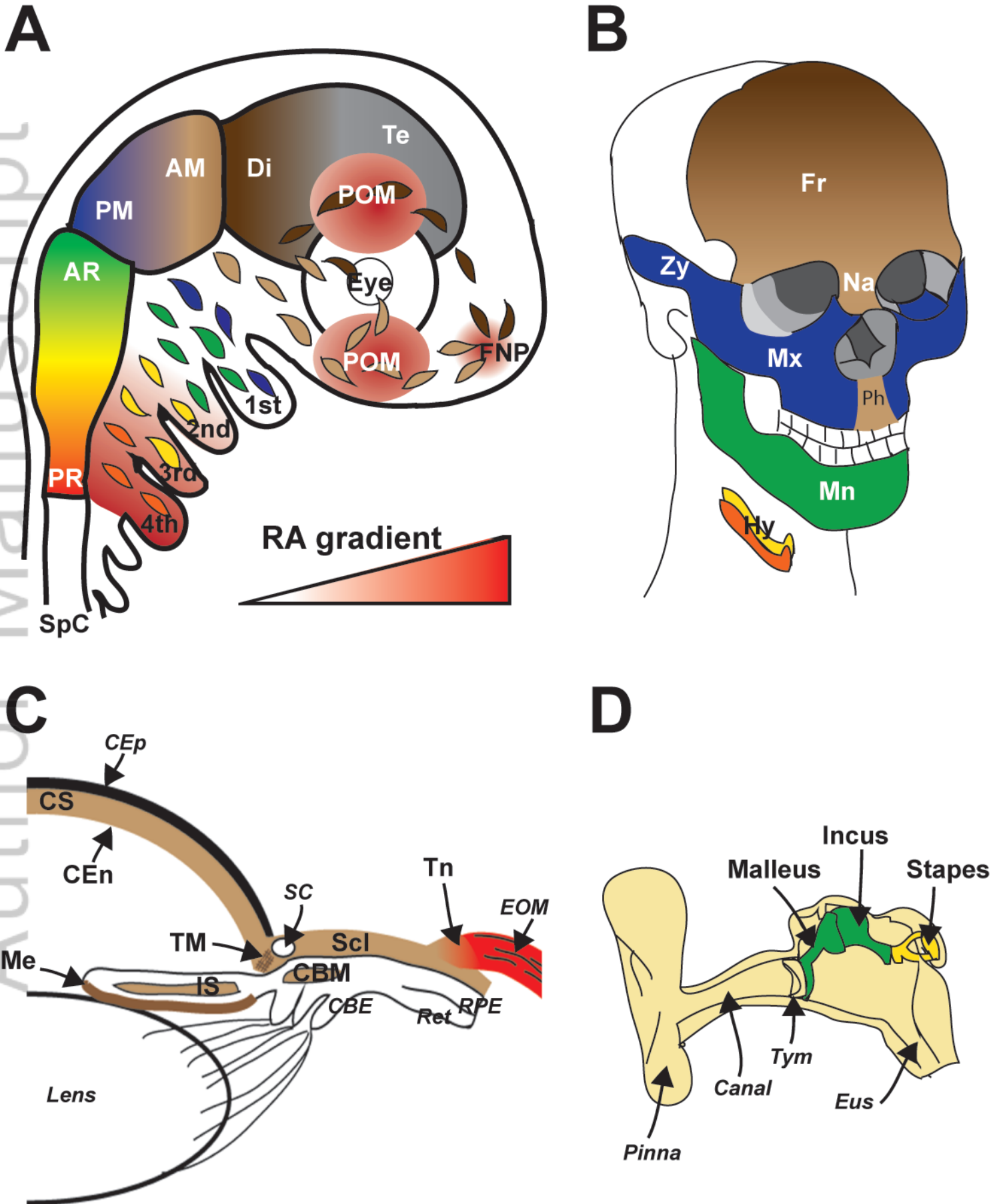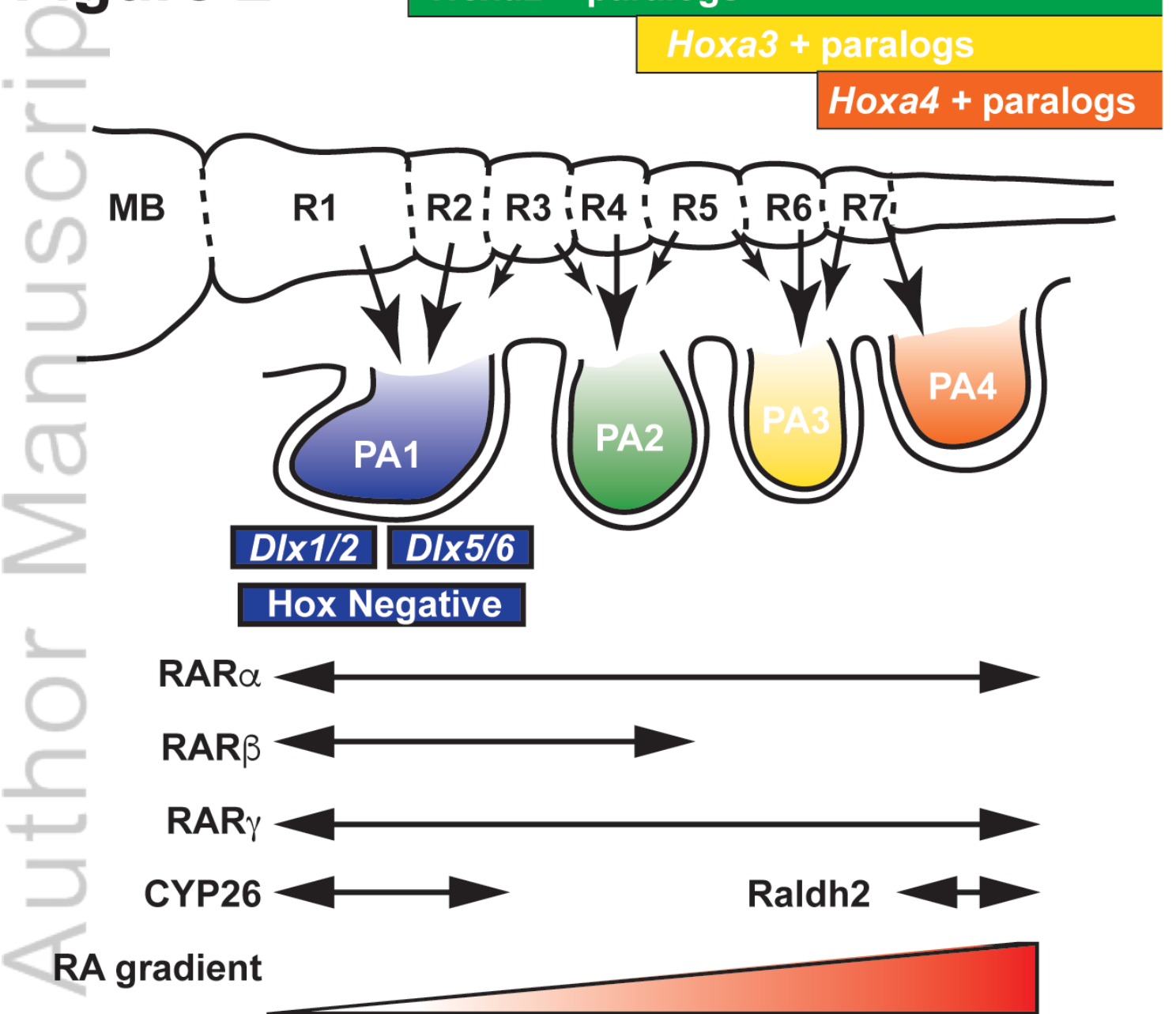
Figure 1

Figure 2

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