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Mechanisms of retinoic acid signalling and its roles in organ and limb development

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Abstract

Retinoic acid (RA) signalling has a central role during vertebrate development. RA synthesized in specific locations regulates transcription by interacting with nuclear RA receptors (RARs) bound to RA response elements (RAREs) near target genes. RA was first implicated in signalling on the basis of its teratogenic effects on limb development. Genetic studies later revealed that endogenous RA promotes forelimb initiation by repressing fibroblast growth factor 8 (*Fgf8*). Insights into RA function in the limb serve as a paradigm for understanding how RA regulates other developmental processes. *In vivo* studies have identified RAREs that control repression of *Fgf8* during body axis extension or activation of homeobox (Hox) genes and other key regulators during neuronal differentiation and organogenesis.

Vitamin A (retinol) is an important nutrient for adult health[1,2] and embryonic development[3,4]. Early studies identified active metabolites of vitamin A, such as 11-*cis*-retinaldehyde (a light-absorbing molecule that is important for vision)[5] and all-*trans*-retinoic acid (RA; commonly known as retinoic acid)[6]. Although the function of RA was unclear, it had been observed that RA administration had teratogenic effects on limb development[7–9]. Later studies revealed that RA controls gene expression directly at the transcriptional level through nuclear RA receptors (RARs) that bind to RA response element s (RAREs)[10,11]. They also showed that RA mediates the growth and development functions of vitamin A[12]. RA regulates development by acting as a diffusible signalling molecule that controls the activity of a family of RARs (reviewed in REFS 13,14). Moreover, RA is essential in adults for maintenance of epithelial homeostasis[15], and for spermatogenesis[16], immune function[17] and brain function[18].

The ability of RA to stimulate cellular differentiation has been exploited for the treatment of cancer and in regenerative medicine to guide embryonic stem cell differentiation[19]. Determining the function of RA during normal development is challenging because pharmacological studies (treating embryos or cell lines with exogenous RA or RAR antagonists) and genetic loss-of-function studies (targeting RA synthesis in embryos) often

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give conflicting results. However, the identification of direct target genes of endogenous RA has provided insights into the molecular mechanisms underlying RA signalling *in vivo*.

In this Review, we discuss the current knowledge of RA function during organ and limb development, focusing on genetic studies that have identified genes that are direct targets of RA signalling — such genes have nearby functional RAREs and require endogenous RA for normal expression during embryogenesis.

Mechanisms of RA signalling

RA signalling is dependent on cells that have the ability to metabolize retinol to RA. RA-generating cells release RA, which is taken up by neighbouring cells. Thus, RA signalling is generally considered as paracrine[14], although RA functions in an autocrine manner during spermatogenesis[16]. Signal transduction involves binding of RA to a nuclear RAR, which forms a heterodimer complex with retinoid X receptor (RXR). RAR–RXR modulates transcription by binding to DNA at a RARE located in enhancer regions of RA target genes[13,14] (FIG. 1).

Transcriptional regulation

In the classical model of RA-dependent gene activation, unliganded RAR–RXR heterodimers bind to RARE sequences and repress transcription of their associated genes, unless activated by RA binding[20–23]. However, additional co-regulators and epigenetic changes contribute to transcriptional regulation. In the repressive unliganded state, the RAR–RXR heterodimer recruits co-repressors such as nuclear receptor co-repressor 1 (NCOR1) and NCOR2 (also known as SMRT), which in turn recruit histone deacetylase (HDAC) protein complexes and Polycomb repressive complex 2 (PRC2). This results in histone H3 lysine 27 trimethylation (H3K27me3), chromatin condensation and gene silencing[24–26] (FIG. 1a). RA binding to RAR–RXR induces a conformational change in the heterodimer, which promotes the replacement of repressive factors by co-activators such as nuclear receptor co-activator 1 (NCOA1; also known as SRC1), NCOA2 (also known as SRC2) or NCOA3 (also known as SRC3). These co-activators recruit histone acetylase (HAT) complexes and Trithorax proteins, which mediate H3K4me3, chromatin relaxation and gene activation[27,28] (FIG. 1a). During activation, co-activators bind to RAR, not RXR, which is consistent with RA-liganded RAR being the key regulatory component of RAR–RXR heterodimers[29]. However, there are exceptions to the classical model: RARE sequences upstream of fibroblast growth factor 8 (*Fgf8*) and homeobox B1 (*Hoxb1*) mediate gene repression, rather than activation, because RA binding to RAR leads to the recruitment of PRC2 and HDAC, and triggers H3K27me3 (REFS 30,31) (FIG. 1b).

Functional RAREs near genes that require RA for normal expression during development typically consist of hexameric direct repeats (DRs) — (A/G)G(T/G) TCA — with interspacing of 5 bp (DR5 elements) or 2 bp (DR2 elements) (TABLE 1), unlike vitamin D and thyroid hormone response elements, which typically exhibit DR3 and DR4 configurations, respectively[32,33]. Other hexameric repeat configurations have been found to bind to RARs in cell line studies involving chromatin immunoprecipitation followed by sequencing (ChIP–seq)[34], but their *in vivo* importance is unknown. The DR1 element has

been suggested to function as a RARE on the basis of *in vitro* studies in which RA function was forced by high levels of exogenous RA and overexpression of RAR[35], but there is so far no evidence for DR1 elements controlling RA signalling *in vivo* (TABLE 1). DR1 elements are required for signalling by other nuclear receptors, such as peroxisome proliferator-activated receptor (PPAR), chicken ovalbumin upstream promoter (COUP) transcription factors and hepatocyte nuclear factor 4 (HNF4)[36].

For both DR5 and DR2 elements, the 5′ half-site binds to RXR and the 3′ half-site binds to RAR[33], with AGTTCA being the most efficient hexamer for RAR binding[37] (FIG. 1). It is unclear what determines whether a RARE has an activating or a repressing function. The repressive RAREs upstream of *Fgf8* and *Hoxb1*, as well as near zebrafish *POU domain, class 5, transcription factor 3* (*pou5f3*; the homologue of mammalian *OCT4* (also known as *POU5F1*)) and mouse *Wnt8a*, are DR2 elements, but many activating RAREs also have DR2 spacing, whereas others have DR5 spacing (TABLE 1). Thus, even if DR2 spacing is required for repressive RAREs, RARE modality seems to require other factors, such as nearby partner enhancer elements or specific co-regulators.

Three RAR (RARα, RARβ and RARγ) and three RXR (RXRα, RXRβ and RXRγ) isotypes with differing expression patterns play a part in controlling the dynamics of RA signal transduction, although RARα, RXRα and RXRβ are ubiquitously expressed in mouse embryos[38,39]. There is a marked functional redundancy between the RAR and RXR isotypes, with only RXRα-null mice exhibiting embryonic lethality[13]. Thus, nuclear-receptor abundance is likely to be secondary to RA availability as the main determinant that drives RA signalling.

RA availability

RA abundance is governed by a tissue-specific enzymatic network in which retinol dehydrogenases (RDHs) and retinaldehyde dehydrogenases (RALDHs) synthesize RA, while cytochrome P450 26 (CYP26) proteins degrade RA[13,14] (BOX 1). Diffusion of RA generates gradients of RA signalling activity that are distributed in a dynamic spatiotemporal manner, as detected by the RA-reporter transgenic mouse *RARE–lacZ*[40] (FIG. 1c) or by RA-reporter strains of zebrafish[41,42]. During early development of vertebrate embryos, a two-tailed gradient of RA activity is established, with trunk mesoderm as the source and with activity declining anteriorly towards the hindbrain and heart, as well as posteriorly towards the caudal progenitor zone, where RA is degraded[40,42] (BOX 1). These gradients of RA activity combine with opposite gradients of FGF8 activity generated in the heart[43] and caudal progenitor zone[44] to create important control mechanisms during body axis extension. In addition, as limbs develop, RA generated by RDH10 and RALDH2 (also known as ALDH1A2) in the trunk enters the limb proximal region but is withheld distally by the action of CYP26B1 (BOX 1).

RA function during limb development

The presence of RA in the proximal but not distal limb suggests that it may have a role in limb proximodistal patterning, but studies in chick and mouse embryos have resulted in different models of RA function in limbs (FIG. 2).

Two-signal model for limb patterning

Limb patterning across the anteroposterior axis (thumb to little finger) and the proximodistal axis (upper arm (stylopod) to lower arm (zeugopod) to hand (autopod)) can be significantly altered by exogenous RA treatment, and such experiments were the first to suggest that RA might function as a signalling molecule. In mice, RA treatment has a teratogenic effect on chondrogenesis of the limb skeleton, resulting in stunted limbs[7]. RA-treated chick limbs exhibit anteroposterior duplications, whereas RA treatment of adult amphibian limbs while they are regenerating results in proximo-distal duplications[8,9]. A later study reported that the chick embryonic limb proximodistal axis is also sensitive to high levels of exogenous RA when it is applied to distal limb regions (which are normally devoid of RA activity); this causes distal expression of the proximal-specific *Meis1* and *Meis2* genes (*Meis1/2*), thus inhibiting distal-limb development. Conversely, treatment of proximal limbs with either RAR antagonists or FGF8 downregulates the expression of *Meis1/2*, suggesting that RA–FGF8 antagonism controls limb proximodistal patterning[45].

Follow-up studies in chicks using limb engraftment techniques suggested that the treatment of distal limb tissue with endogenous concentrations of RA can still affect proximodistal patterning[46,47]. Notably, engraftment of distal limb slices to sites of endogenous RA activity (somites), combined with repression of FGF signalling, results in ectopic *Meis1* activation, whereas *ex vivo* culture of very early limb mesenchyme in 25 nM of RA (along with FGF and WNT growth factors) prevents both *Meis1* downregulation and expression of distal-limb markers[46,47].

Together, these studies support a ‘two-signal hypothesis’, whereby proximal RA activity promotes stylopod specification through the activation of *Meis1/2* expression, whereas distal FGF signals emanating from the apical ectodermal ridge (AER) antagonize *Meis1/2* expression and specify more-distal structures (zeugopod and autopod) (FIG. 2e). A recent chick study has expanded on this model and suggests that there is a two-tier RA dose–response across the proximodistal axis, patterning both the proximal compartment (high RA) and more-distal compartments (low RA)[48].

Genetic studies suggest that RA is dispensable for limb patterning

Genetic loss-of-function studies in mouse verified that distal-limb FGF signalling (primarily FGF8) is required for *Meis1/2* repression and normal limb proximodistal patterning[49]. However, genetic abrogation of limb RA activity in conditionally rescued mouse *Raldh2*$^{-/-}$ embryos (treated with a low dose of RA at embryonic day 7.5 (E7.5) to avoid early lethality) or in untreated *Rdh10*$^{trex/trex}$ point mutants revealed no effect on hindlimb proximodistal or anteroposterior patterning[50–52]. Notably, whereas *Rdh10* mutants display forelimb stunting, their hindlimbs are normal, and patterning markers (including *Meis1/2*) remain unaffected in both forelimbs and hindlimbs[52,53]; this suggests that loss of RA causes a forelimb-specific limb initiation defect, which casts doubt on the hypothesis that RA is required during limb patterning (FIG. 2a,b). Earlier studies had reported that conditionally rescued mouse *Raldh2*$^{-/-}$ embryos also exhibit stunted forelimbs that still maintain proximal *Meis2* expression[51,54].

It has been suggested that *Rdh10*$^{trex/trex}$ point mutants might have trace amounts of RA undetected by the *RARE– lacZ* (RA) reporter that could control proximodistal patterning[48]. However, the RDH10-trex mutant protein has no detectable retinol-oxidizing activity[50]. Moreover, it has been recently reported that *Rdh10*$^{-/-}$ knockout and *Rdh10*$^{trex/trex}$ mice have similar phenotypes, suggesting that previously observed differences were attributable to genetic background. Both strains have normal hindlimbs and stunted forelimbs at E10.5, and no RA activity was detected in any limbs using the *RARE–lacZ* reporter. Both strains display variable survival from E10.5 to E14.5, and survival is likely to be dependent on variable neuroectodermal RA activity[55]. Neural-specific RA synthesis might be catalysed by another retinol-metabolizing enzyme such as epidermal retinol dehydrogenase 2 (RDHE2; also known as SDR16C5)[56]. *RARE–lacZ* expression in the mesoderm of *Rdh10* mutants can be activated by treatment with 0.25 nM of RA[53], which is ~100-fold less than the level of endogenous RA in mouse limb buds (30 nM)[57]. This high sensitivity of the *RARE–lacZ* reporter system suggests that there is at least a 100-fold reduction in endogenous limb RA activity in *Rdh10* mutants. Such a reduction of RA activity should affect patterning if RA was required.

Off-target effects of RA or RAR antagonist treatments

Rdh10$^{trex/trex}$ embryos cultured *ex vivo*, and treated with 10 μM of the same RAR antagonist (BMS493) used for chick studies[45,46], maintained normal proximal-limb *Meis1/2* expression, thus ruling out the possibility that residual RA activity in *Rdh10* mutants could be sufficient to pattern the limb proximodistal axis[53]. These findings conflict with chick studies, in which beads soaked with RAR antagonist and implanted in the limb led to *Meis1/2* downregulation[45,46,48]. However, the beads were soaked in very high concentrations of the drug, leading to potential off-target effects in tissues close to the bead. As BMS493 functions as an inverse agonist that silences genes in the vicinity of RAR-bound RAREs[58], high drug concentrations might dominantly switch off numerous genes that happen to have a RARE nearby but that normally use other regulatory elements under physiological RA conditions. Recent RAR ChIP studies and *in silico* analyses have discovered 13,000–15,000 potential RAREs[34,59], most of which have not been attributed to endogenous RA signalling, but many of which might become off-targets during treatment with high amounts of RA or RAR antagonists.

Reconciling findings in chicks and mice is an ongoing challenge. RA treatment seems to be capable of ectopically activating *Meis1/2* expression in the distal limb, whereas RA localization in the *Meis1/2* expression domain (proximal limb) suggests a role in proximodistal patterning. However, genetic studies show that neither endogenous *Meis1/2* expression nor hindlimb patterning requires RA in mice. Could mouse forelimbs, which are stunted in *Rdh10* mutants, require RA for proximodistal patterning? This seems unlikely because patterning markers are still expressed in forelimbs of *Rdh10* mutants (for example, *Meis2* is still robustly expressed in the proximal region of the stunted forelimb even though the domain is smaller[52]); moreover, pharmacological and RA gain-of-function experiments in chicks[45,47] and mice[60] affect both forelimbs and hindlimbs. One possibility is that mice and chicks use different mechanisms to control *Meis1/2* activation, but this can only be

resolved by genetic manipulation of chicks to eliminate RA synthesis, thus providing results that are not subject to the potential off-target effects of RA or RAR antagonist treatments.

RA degradation is required to prevent RA teratogenesis

Cyp26b1 expression is induced by distal FGFs expressed in the limb AER to degrade RA in the distal limb[61]. In *Cyp26b1*$^{-/-}$ mouse embryos, RA degradation is impaired, leading to overexposure of the limb to RA throughout its development[60] (FIG. 2a,c). In these mutants, *Meis1/2* expression extends to the distal limb, which is consistent with chick RA-treatment studies. However, forelimbs and hindlimbs are truncated along the entire proximodistal axis, which is inconsistent with RA functioning as an inducer of proximal identity. Further studies revealed that teratogenic mechanisms, such as increased apoptosis and a block in chondrogenic differentiation, cause the limb defects seen in *Cyp26b1*$^{-/-}$ mice, overshadowing the effects of disrupted patterning of molecular markers[60,62,63]. *Cyp26b1*$^{-/-}$*Rarg*$^{-/-}$ double-mutant embryos display a partial rescue of *Cyp26b1*$^{-/-}$ limb truncations, indicating that RARγ is important for RA-induced teratogenicity[62]. RARγ and RARα function in an unliganded state to promote chondrocyte differentiation, as well as cartilage and bone growth, and such functions are disrupted by exogenous RA exposure[22,64]. These observations indicate that CYP26B1-dependent RA degradation in the distal limb is required for RA-independent functions of RARγ and RARα in chondrocyte development and bone growth but not for directing proximodistal patterning. Moreover, RA presence in the proximal limb does not necessarily correlate with a biological function: it might result as diffusion overflow from the trunk, where RA is required during forelimb initiation (see below).

One-signal model for limb patterning

The most parsimonious RA-independent limb proximodistal patterning model in mice is a one-signal model that is driven by distal FGF and involves homeobox (Hox) gene-mediated autonomous mechanisms (FIG. 2d). In this model, FGFs drive outgrowth and activate *Cyp26b1* to remove RA distally and thus prevent limb teratogenesis (FIG. 2d). Additionally, genetic studies indicate that distal FGF signals are required to repress *Meis1/2* (REF. 49). However, FGFs are not detected in early limb buds before AER formation[65], resulting in early expression of *Meis1/2* throughout the whole limb, which establishes the proximal limb (stylopod). After the AER is established, it can be reasoned that *Meis1/2* expression is repressed by distal FGFs as the zeugopod forms, creating the junction between the stylopod and zeugopod.

Specification of each of the three proximodistal limb segments depends on a different set of Hox genes, which are activated in a temporally collinear manner to drive the progressive specification of different limb segments[66]. *Meis1/2*, controlled by FGF, might refine HOX activity by controlling the nuclear localization of pre-B cell leukaemia homeobox (PBX) transcription factors in the proximal limb, which act as both HOX cofactors and regulators of Hox gene activation[67,68]. It has been suggested that *Meis1/2* (perhaps in combination with *Hox9* and *Hox10* genes) provides the patterning code for a basic ancestral single-segment appendage (the stylopod)[69] and that this pattern is then superseded by autonomous

activation of successive *Hox11–13* genes (progressing towards the 5′ ends of each Hox cluster) in two phases to progressively generate the zeugopod and then the autopod.

Recent studies on limb regeneration in salamanders support a progress-zone specification model that is reliant on Hox gene collinearity to explain how proximodistal identity is established as the limb regenerates[70]. At sites of limb amputation, the wound blastema generates RA distally rather than proximally[71], suggesting that RA functions to maintain the blastema rather than to control proximodistal patterning. One notable property of regenerating salamander limbs is their scar-free healing, which is presumably important for regenerating fully functional limbs. Heart regeneration in zebrafish also involves RA synthesis through upregulation of *raldh2* (also known as *aldh1a2*), which might prevent scar formation[72], perhaps through modulation of the immune response[17]. Thus, the presence of RA signalling in the wound blastema during limb regeneration is consistent with roles in proliferation and prevention of scar formation but not necessarily proximodistal patterning. The ability of RA treatment to alter proximodistal patterning of regenerating limbs[9] could be attributed to off-target effects of high amounts of RA that alter normal blastema function.

An RA requirement during forelimb initiation

Administration of the RA-synthesis inhibitor disulfiram before wing bud establishment in chick embryos prevents wing bud formation[73], and vitamin A-deficient rat embryos display forelimb hypoplasia[74]. Subsequent genetic studies reported that *Raldh2*$^{-/-}$ mouse embryos, which lack detectable RA signalling, fail to initiate forelimb development (which normally begins at E8.5, approximately 12 hours before embryonic lethality in this mutant)[12,51]. Consistently, zebrafish mutants lacking *raldh2* do not develop pectoral fins[75,76]. Both mouse and zebrafish *Raldh2* mutant embryos lack expression of T-box 5 (*Tbx5*) in the presumptive forelimb field of the lateral plate mesoderm. *Tbx5* is essential for forelimb initiation[77–79] because it stimulates epithelial--to-mesenchymal transition[80] and activates *Fgf10* expression, which is needed for early outgrowth[81].

Further insight was achieved through comparison of *Raldh2*$^{-/-}$ and *Rdh10* mouse mutants, which revealed a tight association between the distribution of RA activity, the restriction of body axis *Fgf8* expression and the activation of *Tbx5* (REF. 53). Immediately before limb *Tbx5* activation, RA is normally present throughout the trunk, where it acts to repress and confine *Fgf8* expression to rostral and caudal domains (the heart and caudal progenitor zone) on either side of the forelimb field (FIG. 3a). In *Rdh10* mutants, RA activity is lost in the heart and forelimb domains but is maintained in the caudal neuroectoderm (as a result of activity by an unidentified RDH)[53]. As such, *Rdh10* mutants exhibit an ectopic *Fgf8* expression domain that expands posteriorly from the heart into the emergent forelimb field, with forelimb *Tbx5* expression both delayed and significantly shortened along the antero-posterior axis[53] (FIG. 3b). In *Raldh2*$^{-/-}$ embryos, which lack RA activity in both mesoderm and neuroectoderm, ectopic *Fgf8* expression enters the forelimb field from both the heart and the caudal progenitor zone, with limb *Tbx5* expression completely failing[51] (FIG. 3c). This comparison indicates that the underlying cause of stunted (or absent) forelimb growth in RA-synthesis mutants is excessive FGF8 activity: such activity perturbs the activation of *Tbx5*, the expression of which demarcates the forelimb field. This model is also supported

by the positive correlation seen between forelimb size and amount of maternal RA administration in RA-rescued *Raldh2*$^{-/-}$ mutants[54].

In zebrafish, ectopic Fgf signalling enacted through the expression of constitutively active Fgf receptor 1 (Fgfr1) reduces *tbx5* expression in limbs and stunts pectoral fin development[82]. Similarly, in cultured E8.25 mouse embryos, the addition FGF8 blocks the activation of forelimb-field *Tbx5* expression[53]. Together, these studies suggest that RA is required in the forelimb field to neutralize the negative influence of trunk FGF signalling on *Tbx5* induction. This has been functionally tested in zebrafish *raldh2* mutants and wild-type zebrafish treated with the RA-synthesis inhibitor diethylaminobenzaldehyde (DEAB), whereby genetic or pharmacological reduction of Fgf signalling can rescue pectoral fin development[51,53,83]. Thus, RA antagonism of *Fgf8* along the primary body axis, both anterior and posterior to the forelimb field domain, is required to permit normal spatiotemporal activation of forelimb *Tbx5* (FIG. 3d). *Fgf8* is a direct target of RA signalling because it has a RARE that is required for the repression of *Fgf8* expression *in vivo*[30] (TABLE 1).

Hindlimb initiation in mice is not perturbed by loss of RA activity in the hindlimb field[50,52]. In axolotl embryos, hindlimbs do not express an RA-reporter transgene, whereas forelimbs do[71]. Hindlimbs typically initiate later than do forelimbs (with a 0.5-day interval in mice), providing one clue to the difference in RA requirement between forelimbs and hindlimbs. In mice, hindlimb initiation occurs at around E9.0, after the primitive streak has regressed and beyond the time that *Fg f8* in the caudal progenitor zone is under the influence of RA[84]. Thus, unlike in the forelimb, loss of RA synthesis should not directly disturb hindlimb initiation through a mechanism involving ectopic *Fgf8* expression. Furthermore, hindlimbs do not express *Tbx5* but instead rely on a combination of *Tbx4*, paired-like homeodomain transcription factor 1 (*Pitx1*) and *Isl1* expression[85–87], which are unaffected in the hindlimbs of RA-synthesis mutants[51,52].

Digit formation requires RA

Late in limb development, RA is required for digit formation, but in this case RA functions to activate key genes. During this time, interdigital *Rdh10* and *Raldh2* expression is activated, yielding prominent interdigital RA activity, as detected by *RARE– lacZ*. This activity is necessary for interdigital tissue loss, as shown by *Rarb*$^{-/-}$*Rarg*$^{-/-}$ double mutants, as well as by *Raldh2*$^{-/-}$ and *Rdh10* mutants[52,88,89]. These studies demonstrated that retention of interdigital tissue is due to loss of apoptosis and is associated with downregulation of the expression of transglutaminase 2 (*Tgm2*) and matrix metalloproteinase 11 (*Mmp11*), which have functional RAREs that require RA for activation (TABLE 1).

RA control of body axis extension

Vertebrate embryos develop in a head-to-tail manner, partly from a bipotential axial (neuromesodermal) stem cell population that is located at the caudal end of the embryo. This cell population generates somitic mesoderm (musculoskeletal progenitors) and posterior neuroectoderm (hindbrain and spinal cord)[90]. Loss of RA synthesis in vitamin A-deficient

avian embryos[44], disulfiram-treated chick embryos[91] and mouse *Raldh2*$^{-/-}$ embryos[92,93] results in somite and posterior neural defects that are associated with encroachment of caudal *Fgf8* expression into trunk tissue; earlier studies showed that treatment of trunk tissue with FGF8 inhibits somitogenesis[94] and neurogenesis[95]. Thus, RA–FGF8 antagonism has a crucial role during mouse and chick body axis extension that is similar to its role in forelimb initiation, with *Raldh2* and *Rdh10* expressed in trunk mesoderm generating RA that diffuses caudally to restrict *Fgf8* expression to the caudal progenitor zone (FIG. 4). In mouse embryos, RA represses *Fgf8* at the epiblast–neural plate border to limit the anterior extent of FGF8 activity[93], while *Fgf8* induces *Cyp26a1* caudally to limit the posterior extent of RA activity[96]. Thus, caudal CYP26A1 functions in a similar way to distallimb CYP26B1 by removing unwanted RA that is teratogenic. Whereas *Cyp26a1* has a functional RARE that allows activation by excess RA in many tissues as a defence mechanism[97], basal *Cyp26a1* caudal expression does not require RA[98] but does require FGF signalling[96]. In zebrafish, caudal *cyp26a1* also functions to eliminate teratogenic RA in the caudal mesodermal progenitor niche[99]. However, in zebrafish, RA does not repress caudal *fgf8* expression to control somitogenesis or neurogenesis, although RA does repress cardiac *fgf8* during pectoral fin (forelimb) initiation[83]. During body axis extension, RA activates genes for posterior neurogenesis in zebrafish in a similar way to that in mice[75,76], and RA directly represses the zebrafish pluripotency regulator *pou5f3* through a nearby RARE as cells exit the caudal progenitor zone[100].

RA–FGF8 antagonism controls somitogenesis

A caudal-high gradient of FGF8 and FGF4 activity in presomitic mesoderm plays an essential part in somitogenesis by providing spatial information that allows somites to form at a position where FGF-induced cell motility has declined to a low level[101,102]. Loss of RA activity in avian and mammalian embryos leads to expanded caudal *Fgf8* expression and the formation of small somites that exhibit left–right asymmetry[44,91–93]. The mechanism through which RA controls somite bilateral symmetry is known to require an interaction between the transcriptional co-regulator arginine–glutamic acid dipeptide repeats protein (RERE) and FGF8 signalling. *Rere* mutants exhibit left–right asymmetric caudal *Fgf8* expression and also show reduced *Rarb* expression, resulting in lower RA signalling[103]. In addition, RERE has been shown to directly regulate the *Fgf8* gene[30]. Thus, RA–FGF8 antagonism, controlled in part by RERE, is a critical determinant of somitogenesis that establishes the appropriate caudal FGF8 gradient to limit motility in the presomitic mesoderm, which is needed for somite formation.

Caudally expressed *Cyp26a1* controls RA degradation, which is necessary to prevent caudal teratogenesis and premature termination of somitogenesis[104,105]. At the trunk–tail transition, expression of a specific set of caudal-type homeobox (Cdx) and Hox genes also functions to maintain axis extension by stimulating expression of *Wnt3a* and *Cyp26a1* (REF. 106), probably through WNT-mediated upregulation of *Fgf8* expression, which in turn upregulates *Cyp26a1* expression[96]. Studies in chicks (which have shorter tails than mice) suggest that somitogenesis is terminated soon after the trunk–tail transition by upregulation of caudal *Raldh2* expression, which downregulates caudal *Fgf8* expression[107]. In mouse embryos, which have much longer tails with more somites, caudal *Raldh2* expression was suggested

to function in a similar way[108]. However, studies on conditionally rescued *Raldh2*$^{-/-}$ mouse embryos have shown that *Raldh2* expression in tail somites does not generate RA activity that is detectable by *RARE–lacZ*, and *Raldh2* is not required for the termination of caudal *Fgf8* expression or somitogenesis[84]. These studies showed that *Raldh2* expression alone does not guarantee RA synthesis: an upstream enzyme that metabolizes retinol to retinaldehyde is also needed.

Are any RA target genes other than *Fgf8* required for somitogenesis? Although studies in *Xenopus laevis* suggested a role for RA in activation of mesoderm posterior 2 (*mesp2*) at the somite-forming boundary as a part of the somitogenesis clock mechanism[109], genetic loss-of-function studies do not support such a role for RA in mice[93]. However, RA generated by *Raldh2* is required in mice to activate caudal mesodermal expression of *Cdx1* (by two conserved RAREs) during early somite stages to control Hox gene expression and to prevent vertebral homeotic transformations[110–112].

Mechanism through which RA represses caudal *Fgf8*

Mutational studies demonstrated that a RARE upstream of *Fgf8* is required for RA repression of caudal *Fgf8* in transgenic mouse embryos, thereby showing that RA directly represses *Fgf8* transcription *in vivo*[30]. ChIP studies on *Raldh2*$^{-/-}$ embryos suggested that the mechanism of caudal *Fgf8* repression involves RA-dependent recruitment of PRC2 and HDAC1 to the *Fgf8* RARE and nearby deposition of the repressive H3K27me3 mark[30] (discussed above) (FIG. 1). In addition, RA-dependent movement of the *Fgf8* locus to the nuclear periphery as cells exit the caudal progenitor zone might be associated with repression[113]. Thus, *Fgf8* is a crucial direct target of RA in the caudal progenitor zone, and the repression of *Fgf8* is necessary to obtain normal-sized somites with bilateral symmetry, as well as to prompt neuronal differentiation as neuroectoderm exits the caudal progenitor zone.

RA activation of genes during neurogenesis

Treatment of stem and progenitor cells with RA induces neuronal differentiation *in vitro*[19], suggesting either that RA promotes neuroectoderm specification or that neural cells require RA for differentiation. However, studies with chicks, rats, mice and zebrafish lacking endogenous RA indicate that RA only functions in specific regions along the central nervous system[114–118]. During mouse neurogenesis, RA is first generated during early hindbrain development, after forebrain and midbrain neuroectoderm specification[118]. RA generated by RALDH2 is required for activation of at least ten 3′ Hox genes (which are found in the 3′ portions of each Hox gene cluster) and for activation of HNF1 homeobox B (*Hnf1b*), which represses *Hoxb1* during anteroposterior patterning of the hindbrain and spinal cord (FIG. 4). RA also activates *Cdx1* in caudal neuroectoderm at early somite stages[119], which represses the hindbrain developmental programme in this region to allow spinal-cord specification[120,121]. Moreover, RA is essential for activating neurogenin 2 (*Ngn2*; also known as *Neurog2*) to stimulate neurogenesis and dorsoventral patterning in the spinal cord[122]; paired box 6 (*Pax6*) to stimulate motor neuron differentiation[44]; and developing brain homeobox 1 (*Dbx1*) to promote interneuron differentiation[123]. These genes all have RAREs, many of which are required for RA-dependent activation *in vivo* (TABLE 1).

Activation of *Pax6* and *Dbx1* requires both a RARE and a SOX-binding site located within 50 bp of each other[123]. Moreover, *Pax6* activation depends on caudal RA–FGF8 antagonism, which releases FGF-induced chromatin compaction at the *Pax6* locus in neural cells that are exiting the caudal progenitor zone[113].

Roles of RA during organogenesis

RA signalling has essential roles during the development of some organs, as shown by genetic loss-of-function studies that remove endogenous RA synthesis.

Eye morphogenesis

During mouse eye development, RA is generated in the optic vesicle (an outpocketing of the forebrain) and the optic cup by expression of *Raldh1* (also known as *Aldh1a1*) in dorsal retina and *Raldh3* (also known as *Aldh1a3*) in ventral retina[124], whereas zebrafish express *raldh2* dorsally and *raldh3* ventrally[125]. Early studies suggested that RA controls retina patterning. However, ablation of *Raldh1* and *Raldh3* in mice and pharmacological and genetic knockdown of RALDH function in zebrafish revealed that loss of optic-cup RA activity leads to excessive perioptic mesenchyme growth, which is associated with dysgenesis of the cornea and eyelid, and mechanical stresses that lead to abnormal optic-cup formation[126–128]. Thus, RA generated in the retina functions in a paracrine manner to control development of the surrounding perioptic mesenchyme (FIG. 5a). Further studies indicated that within the perioptic mesenchyme, RA directly activates *Pitx2* expression through a nearby RARE, which in turn induces dickkopf homologue 2 (*Dkk2*) to locally suppress WNT signalling[129]. Thus, RA controls eye morphogenesis by preventing excessive WNT signalling in the perioptic mesenchyme.

Forebrain basal ganglia differentiation

RA generated in the optic vesicle can diffuse to the early forebrain. Studies in chicks suggested that RA might control early forebrain patterning in addition to eye development[130], but mutational studies of *Raldh2*, *Raldh3* and *Rdh10* have shown that endogenous RA is not required for early forebrain patterning in mice[55,131]. However, during later stages of forebrain development, RA is generated in the lateral ganglionic eminence by RALDH3 and in the meninges by RDH10 and RALDH2. Studies using *Raldh3*$^{-/-}$ mice showed that RA diffuses into the forebrain basal ganglia, where it is required for dopaminergic development through the induction of dopamine receptor D2 (*Drd2*), which has a nearby functional RARE[131,132] (FIG. 5b). Moreover, RA stimulates γ-aminobutyric acid (GABA)-ergic neuronal differentiation in the forebrain basal ganglia by stimulating expression of glutamate decarboxylase 67 (*Gad67*; also known as *Gad1*) in the striatum and olfactory-bulb interneurons[133]. However, RA might regulate *Gad67* indirectly because no RARE has been identified nearby.

RA generated by RDH10 and RALDH2 in the meninges was initially proposed to diffuse into the forebrain cortex and stimulate cortical neuron generation on the basis of analysis of *Rdh10* mutants and mutants of forkhead box C1 (*Foxc1*) that reduce meningeal expression of *Rdh10* (REF. 134). However, *Rdh10* mutants also exhibit severe craniofacial defects,

which could complicate analysis of forebrain development[50]. In further studies, *Raldh2* and *Rdh10* mutants that do not exhibit craniofacial defects (owing to a short early rescue protocol with either RA or retinaldehyde) but that still lack RA activity at later stages in the meninges and cortex were found to have normal expansion of the forebrain cortex[55,133]. As no RA target gene has been identified in the cortex, further investigation is needed to determine whether RA generated in the meninges regulates cortical differentiation or whether its role is limited to development of the cranial neural crest during formation of the skull[135].

Coordination between liver and heart development

Studies on heart development using RXRα-mutant mice suggested that RA action in the epicardium stimulates growth of the ventricular myocardium[136,137]. However, analyses of *Raldh2*$^{-/-}$ mice revealed that ventricular growth requires RA in the liver mesothelium but not the epicardium[138]. RA activates erythropoietin (*Epo*) in the embryonic liver, where it locally stimulates erythropoiesis[139]. However, EPO also travels through the blood-stream to the epicardium, where it induces insulin-like growth factor 2 (IGF2), which stimulates cardiac ventricular growth[138,140]. Thus, RA coordinates liver and heart development to stimulate ventricles to thicken at a point in development when greater cardiac output is required to distribute red blood cells throughout the body (FIG. 5c). ChIP studies in fetal liver revealed a RARE near *Epo* that binds to RARs, suggesting that RA directly activates *Epo*[138].

Spermatogenesis and meiotic progression

Female gametes initiate meiosis during fetal ovary development, whereas males delay spermatogenesis and meiosis until after birth. *Cyp26b1* is expressed in developing testes but not ovaries, and studies using *Cyp26b1*$^{-/-}$ embryos and wild-type embryos treated with RA or RAR antagonists suggested that RA generated by RALDH2 in the adjacent mesonephros triggers meiosis in fetal ovaries, whereas CYP26B1 in the testes degrades RA and prevents meiosis[141,142]. However, *Raldh2*$^{-/-}$*Raldh3*$^{-/-}$ embryos initiated meiosis normally in the ovary, despite loss of RA activity in the mesonephros and ovary[143]. By contrast, studies of postnatal conditional *Raldh2*$^{-/-}$ mice demonstrated that male meiosis requires RA activity in spermatocytes to activate expression of the pre-meiotic gene known as stimulated by retinoic acid gene 8 (*Stra8*); *Stra8* is activated by a nearby RARE that was found to bind RARs in ChIP studies in the testis[16]. These findings suggest that endogenous RA is required for male but not female meiosis[144] (FIG. 5d). Other studies have shown that female meiosis in mouse and chick embryos can be stimulated by WNT signalling and progesterone, respectively[145,146]. These studies, as well as those on limb patterning, demonstrate that reliance on *Cyp26b1*$^{-/-}$ mutants or pharmacological studies rather than RA-synthesis mutants can result in misleading conclusions about endogenous RA function.

RA control of *Fgf8* in heart, nasal pit and other sites

As RA directly regulates caudal *Fgf8* expression through a nearby RARE, it is important to know whether RA restricts *Fgf8* expression in other tissues. Analysis of *raldh2*-mutant zebrafish demonstrated that RA restricts the cardiac progenitor pool[147]. Studies of *Raldh2*$^{-/-}$

mice found that RA restricts the size of the second heart field by repressing *Fgf8* expression in the posterior heart mesoderm[43,148], and zebrafish heart development also requires RA–FGF8 antagonism[83]; however, it is currently unknown whether this is a direct effect of RA on *Fgf8* transcription. *Raldh3*$^{-/-}$ studies demonstrated that RA generated by RALDH3 is required to repress *Fgf8* in the olfactory pit for nasal passage formation[131,149]; again, whether RA acts directly is unknown.

RA-treatment studies suggested that RA downregulates *Fgf8* expression in the limb AER[61]; however, genetic studies demonstrated no effect on distal-limb *Fgf8* expression when endogenous limb RA is removed in *Rdh10* mutants[52] or when limb RA is increased in *Cyp26b1*$^{-/-}$embryos[60]. Moreover, treatment of chick embryos with RA or RAR antagonists suggested that RA is needed to activate *Fgf8* expression in the anterior neural ridge within the forebrain[150]; however, complete loss of endogenous RA activity in the heads of *Raldh2*$^{-/-}$*Raldh3*$^{-/-}$ double mutants or *Rdh10* mutants had no effect on forebrain *Fgf8* expression[55,131]. As *Fgf8* has a nearby RARE that is needed for caudal repression by endogenous RA[30], exogenous RA or RAR antagonists might work through this RARE to force off-target or 'off-tissue' effects on *Fgf8* expression in the AER and forebrain that normally do not occur. *Fgf8* is also expressed at the midbrain–hindbrain border (isthmus), generating an anterior-high FGF8 gradient along the hindbrain that opposes the posterior-high gradient of RA along the hindbrain, but RA is not needed to control this *Fgf8* expression domain[53].

RA repression of other signalling pathways

In addition to repressing *Fgf8* expression, RA downregulates other signalling pathways, as shown by studies on *Raldh2*$^{-/-}$ mouse embryos. RA was demonstrated to repress transforming growth factor-β1 (TGFβ1) and *Dkk1* during lung-bud initiation[151,152] and the Sonic hedgehog (SHH) effector GLI-Krüppel family member GLI2 (*Gli2*) during body axis extension[153]; however, as no RAREs were found near these genes, it remains unclear whether they are direct targets of RA repression. Studies of *Raldh2*$^{-/-}$ mice have also shown that RA is required to limit the anterior extent of *Wnt3a* and *Wnt8a* expression in the caudal progenitor zone during body axis extension[119,153]. No RARE has been reported near *Wnt3a*, but RA repression of mouse *Wnt8a* might be direct because it was found, in embryo ChIP studies, to have a nearby DR2 RARE that is able to bind to RARs (TABLE 1).

Conclusions and perspective

Many of the developmental functions of endogenous RA have been revealed, which has led to the discovery of RA target genes harbouring functional RAREs that control gene activation or repression *in vivo*. Although RA function has often been studied using treatment with RA or RAR antagonists, the results of genetic loss-of-function studies that target RA synthesis have shown that a pharma cological approach alone cannot be used to elucidate the function of endogenous RA. A continued search for physiologically relevant direct RA target genes will further reveal the molecular logic that RA signalling uses to control gene activation and repression during development. The genetic loss-of-function studies required to make further progress, including mutation of potential RARE elements,

will be made easier by new techniques such as clustered regularly interspaced short palindromic repeat (CRISPR)–CRISPR-associated 9 (Cas9)-mediated gene editing[154].

Several questions remain to be answered about the mechanism of RA action during limb and organ development. The mechanism of RA–FGF8 antagonism requires a repressive RARE upstream of *Fgf8* that can recruit PRC2 in an RA-dependent manner[30], but how this RARE acts in a repressive versus activating manner is a note worthy topic for further study. During forelimb initiation, the mechanism linking RA–FGF8 antagonism to *Tbx5* activation is unknown. With regard to limb-patterning stages, genetic evidence does not support a two-signal RA–FGF antagonism model for the establishment of proximodistal patterning, so future studies can focus on whether a one-signal distal FGF model is sufficient for driving proximodistal patterning from a progress zone. As RA–FGF8 antagonism is simultaneously required for forelimb initiation, posterior heart formation, neurogenesis, somitogenesis and control of body axis extension from the caudal progenitor zone, it will be interesting to determine whether *Fgf8* repression in all of these locations is mechanistically similar and part of one overall process for controlling anteroposterior boundaries along the primary body axis. Furthermore, among the numerous genes that have been reported to have nearby RAREs that respond to RA treatment in cultured cells[19,34,59], it will be important to determine whether endogenous RA is required for their proper expression in specific tissues *in vivo* and whether this is essential for organogenesis or adult-tissue homeostasis. Such knowledge will be instrumental in guiding regenerative medicine approaches that rely on the treatment of stem cells with RA to generate useful differentiated cells.

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Glossary

RA receptors (RARs)	DNA-binding nuclear receptors that directly regulate transcription in response to binding of their ligand retinoic acid (RA)
RA response elements (RAREs)	DNA elements that bind to retinoic acid (RA) receptors
Caudal progenitor zone	The posterior (caudal) region of vertebrate embryos, which contains axial stem cells and other progenitor cells that progressively differentiate to form the body axis
Body axis extension	The progressive formation of vertebrate embryos in a head-to-tail direction
Stylopod	The proximal element of the limb (that is, upper arm or leg)
Zeugopod	The middle element of the limb (that is, lower arm or leg)
Autopod	The distal element of the limb (that is, hand or foot)

Homeobox (Hox) gene	A cluster of homeobox genes essential for axial patterning that exhibit spatial and temporal collinear expression, with genes in the 3' end of each cluster expressed earlier and more anterior (or proximal for limbs) than 5' genes.
Blastema	A mass of proliferating mesenchymal and epithelial cells that is located at the distal tip of the limb after amputation

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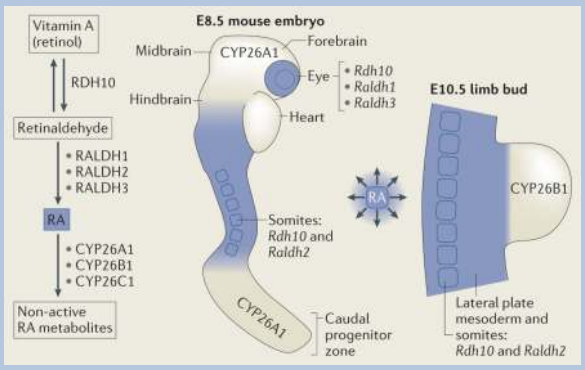
Box 1

RA synthesis and degradation

In mammals, retinoic acid (RA) is initially generated during the late primitive streak stage (embryonic day 7.5 (E7.5) in mice)[118]: retinol dehydrogenase 10 (RDH10)[50,155,156] oxidizes retinol (dietary vitamin A) to retinaldehyde, and retinaldehyde dehydrogenase 2 (RALDH2)[12,157] then oxidizes retinaldehyde to RA (see the figure). Mouse *Raldh2* mutants lack mesodermal and neural RA activity and fail to grow beyond E8.75, whereas *Rdh10* mutants lack mesodermal RA but maintain neural RA activity that allows survival to E10.5-E14.5. *Xenopus laevis* epidermal retinol dehydrogenase 2 (RDHE2; also known as SDR16C5) and zebrafish retinol dehydrogenase 1-like (RDH1L) are additional retinol dehydrogenases that might participate in embryonic RA synthesis[56,158]. *Raldh1* (REF. 159) *and Raldh3* (REF 149) encode additional RALDHs that are expressed beginning at E9.5 or E8.5, respectively, and that are not required for embryonic survival, but these enzymes are required for some developmental processes, such as eye development. A trio of cytochrome P450 RA-degrading enzymes, encoded by cytochrome P450 26A1 (*Cyp26a1*), *Cyp26b1* and *Cyp26c1*, convert RA to inactive forms to halt RA signalling or to prevent unwanted RA signalling in nearby tissues, as RA is freely diffusible[60,104,105,160,161]. Early mouse embryonic expression of genes encoding RA-synthesizing and RA-degrading enzymes are indicated in the figure, and the location of RA activity monitored by the mouse RA response element (RARE)-*lacZ* transgene is shown in blue.



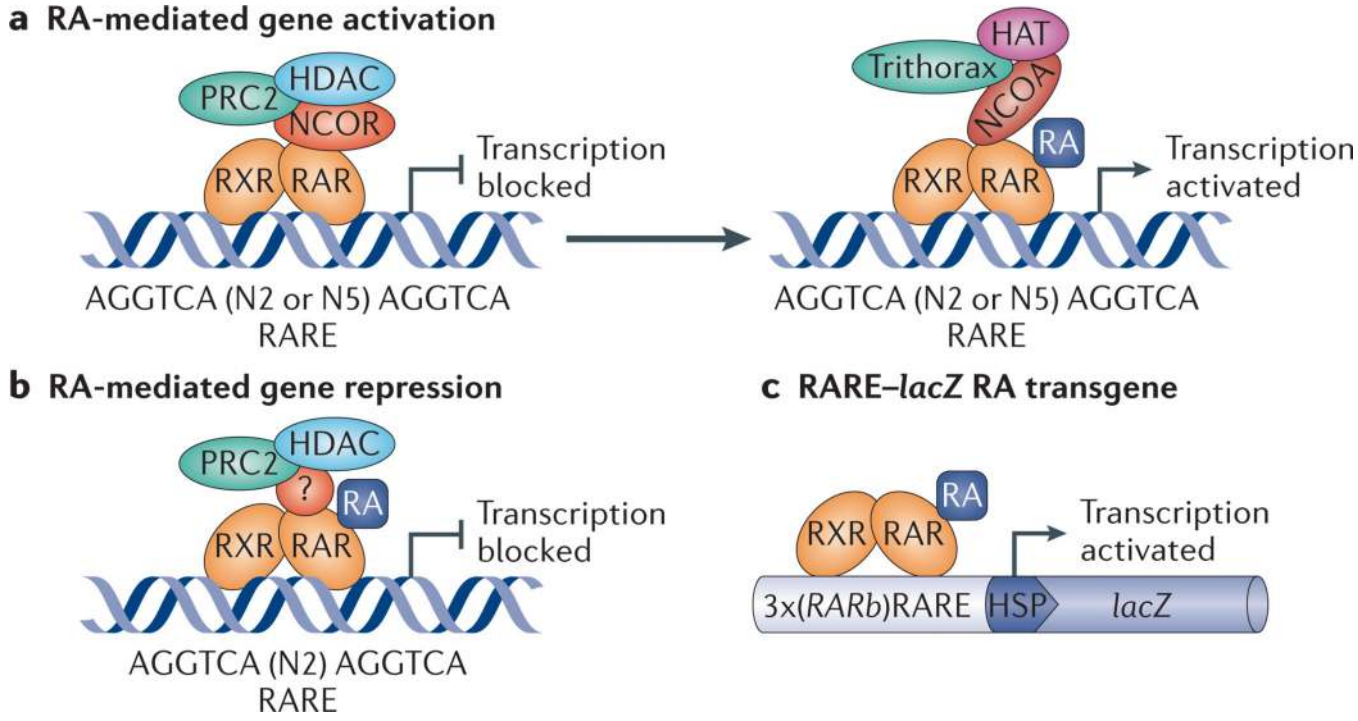


Figure 1. RA signalling mechanism

Retinoic acid (RA) binds to RA receptor (RAR) in an RAR–retinoid X receptor (RXR) heterodimer complex bound to RA response elements (RAREs) near target genes, resulting in control of transcription. **a** | For genes activated by RA, the absence of RA allows co-repressors of the nuclear receptor co-repressor (NCOR) family to bind to RAR and recruit repressive factors such as Polycomb repressive complex 2 (PRC2) and histone deacetylase (HDAC), whereas the presence of RA releases co-repressors and allows co-activators of the nuclear receptor co-activator (NCOA) family to bind to RAR and recruit activating factors such as Trithorax and histone acetylase (HAT). **b** | For genes repressed by RA (such as fibroblast growth factor 8 (*Fgf8*)), the presence of RA allows RAR to recruit PRC2 and HDAC (in this case the co-regulator, if any, is unknown, as indicated by '?'). **c** | The RARE–*lacZ* RA-reporter transgene, which is often used to monitor RA activity *in vivo*, consists of three tandem RAREs (from the *Rarb* gene) located upstream of a basal heat shock promoter (HSP) driving a *lacZ* gene cassette, which leads to expression of β-galactosidase.

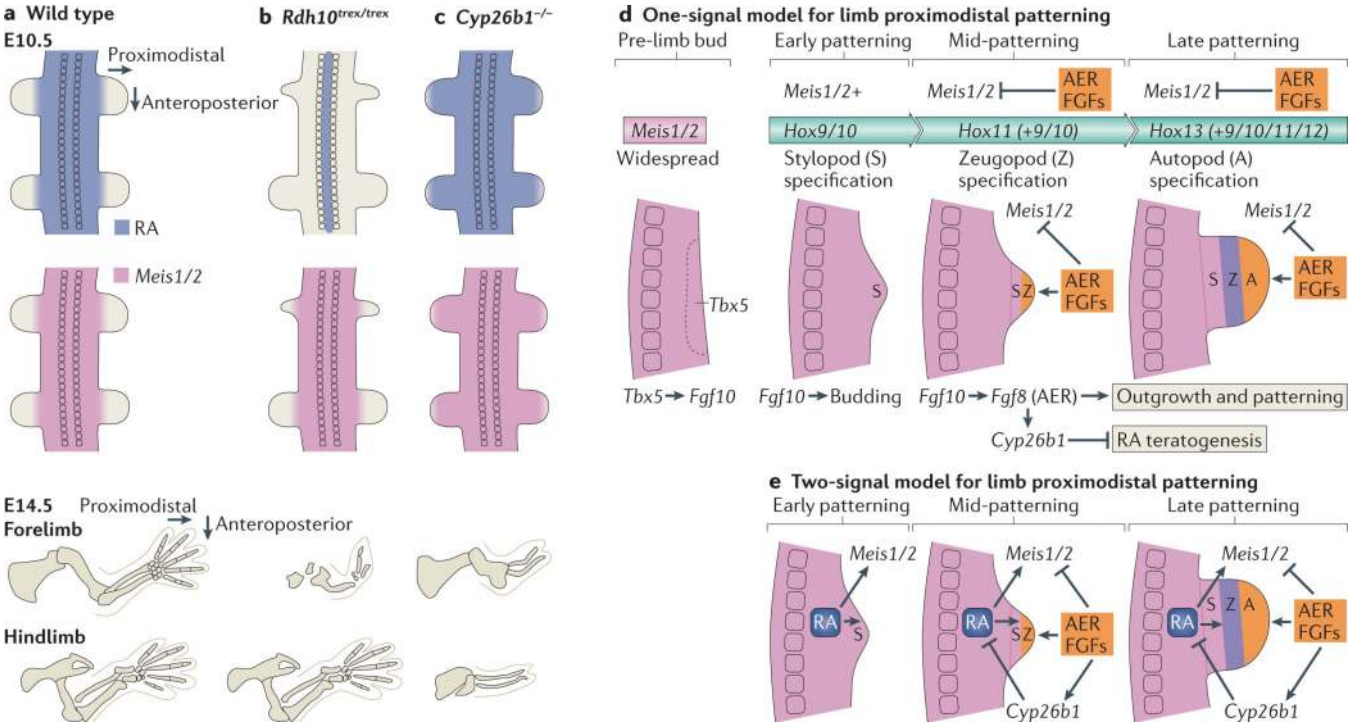


Figure 2. Genetic studies indicate that limb patterning does not require RA signalling but does require FGF signalling and RA degradation

a | During limb-patterning stages, retinoic acid (RA) is prevented from entering the limb by the action of cytochrome P450 26B1 (CYP26B1). At the same time, expression of *Meis1* and *Meis2* (*Meis1/2*) marks the proximal limb. By embryonic day 14.5 (E14.5), all of the skeletal elements of the limbs have formed. **b** | In retinol dehydrogenase 10 (*Rdh10*) mouse mutants (*Rdh10*$^{trex/trex}$), RA is limited to the neural tube and is missing from the limbs, whereas *Meis1/2* expression in the limbs displays a normal distribution during patterning stages. Forelimbs are severely truncated from an early stage, which is indicative of their forelimb initiation defect, whereas hindlimbs have a normal complement of skeletal elements. **c** | In *Cyp26b1*$^{-/-}$ mouse mutants, RA degradation in the limbs is lost, and RA is detectable in more distal limb regions than wild-type mice during limb-patterning stages. *Meis1/2* expression is also extended distally. At E14.5, all limb segments in both forelimbs and hindlimbs are significantly truncated. **d** | Based on mouse genetic studies, we propose a one-signal fibroblast growth factor (FGF)-driven progress-zone model for limb proximodistal patterning coupled with collinear homeobox (Hox) gene activation that does not require RA to specify proximal fate but requires RA degradation to prevent RA teratogenesis. Before limb budding, T-box 5 (*Tbx5*) activates *Fgf10* in the limb field *Fgf10* subsequently activates epithelial-to-mesenchymal transition and proliferation in the limb field, which leads to formation of the limb bud. *Meis1/2* expression is present throughout the early bud and marks stylopod specification, which is dependent on *Hox9* and *Hox10* genes in the forelimb and *Hox10* genes in the hindlimb. Subsequently, activation of *Fgf8* by *Fgf10* in the distal ectoderm leads to formation of the apical ectodermal ridge (AER) and continued distal FGF signalling that promotes outgrowth and activates *Cyp26b1*, which degrades RA to block RA-induced teratogenesis. AER FGF signals also repress *Meis1/2* in the distal limb,

which allows specification of a more distal zeugopod fate. This is dependent on *Hox11* genes, which are activated later and more distally than *Hox9* and *Hox10* genes in an autonomous manner. Later activation of *Hox13* and *Hox12* genes at the distal extremity is required for autopod specification. **e** | Chick studies support a two-signal model for control of limb proximodistal patterning in which RA is required to establish proximal fate, whereas an opposing FGF signal is required for distal fate. RA generated in the trunk diffuses into the proximal limb to stimulate *Meis1/2* expression, and FGF generated distally in the AER then represses *Meis1/2* and induces *Cyp26b1* to degrade RA distally to limit *Meis1/2* expression to the proximal limb.

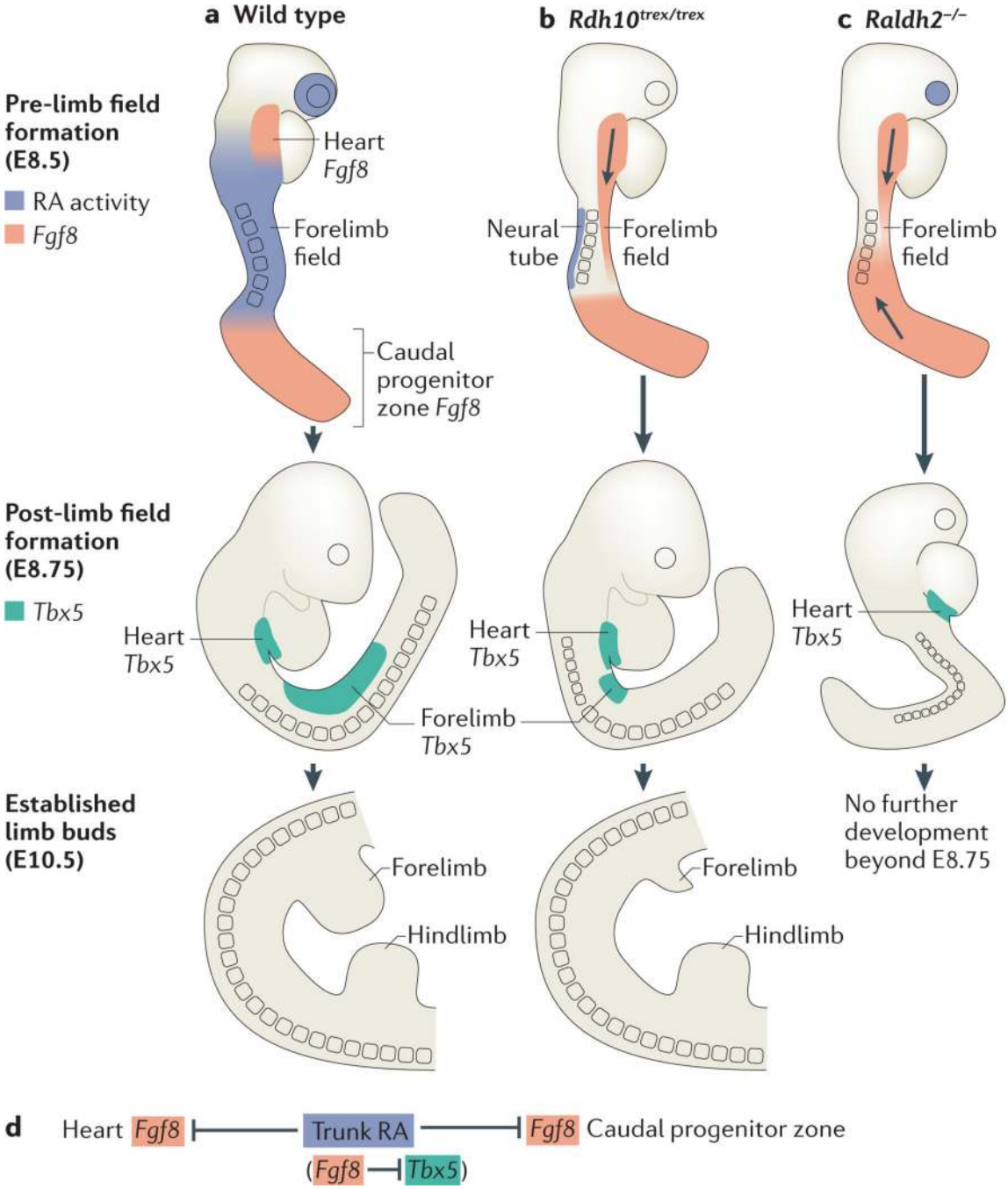


Figure 3. RA– *Fgf8* antagonism regulates the initiation of forelimb budding

a | Before forelimb field formation, wild-type mouse embryos have two domains of fibroblast growth factor 8 (*Fgf8*) expression (red) in the heart and caudal progenitor zone, flanking regions of retinoic acid (RA) signalling in the trunk (blue). T-box 5| (*Tbx5*) expression (green) in the lateral plate mesoderm marks the established forelimb field. By embryonic day 10.5 (E10.5), forelimb and hindlimb buds are clearly established. **b** | In retinol dehydrogenase 10 (*Rdh10*)-mutant mice (*Rdh10*$^{trex/trex}$), loss of mesodermal RA activity is coupled with a posterior extension of cardiac *Fgf8* expression into the

presumptive forelimb field. Limb *Tbx5* expression is subsequently delayed, and the region of *Tbx5* expression is significantly shortened along the anteroposterior axis. At E10.5, forelimbs are hypoplastic, whereas hindlimbs are unaffected. **c** | In retinaldehyde dehydrogenase 2 (*Raldh2*)-mutant mice, a lack of RA activity in the trunk (both mesoderm and neuroectoderm) is coupled with two fronts of ectopic *Fgf8* expression into the presumptive forelimb field, from both the heart and the caudal progenitor zone. Limb *Tbx5* expression is subsequently prevented. *Raldh2*$^{-/-}$ mutants stop developing at around E8.75. **d** | RA–*Fgf8* antagonism is tightly associated with limb *Tbx5* activation.

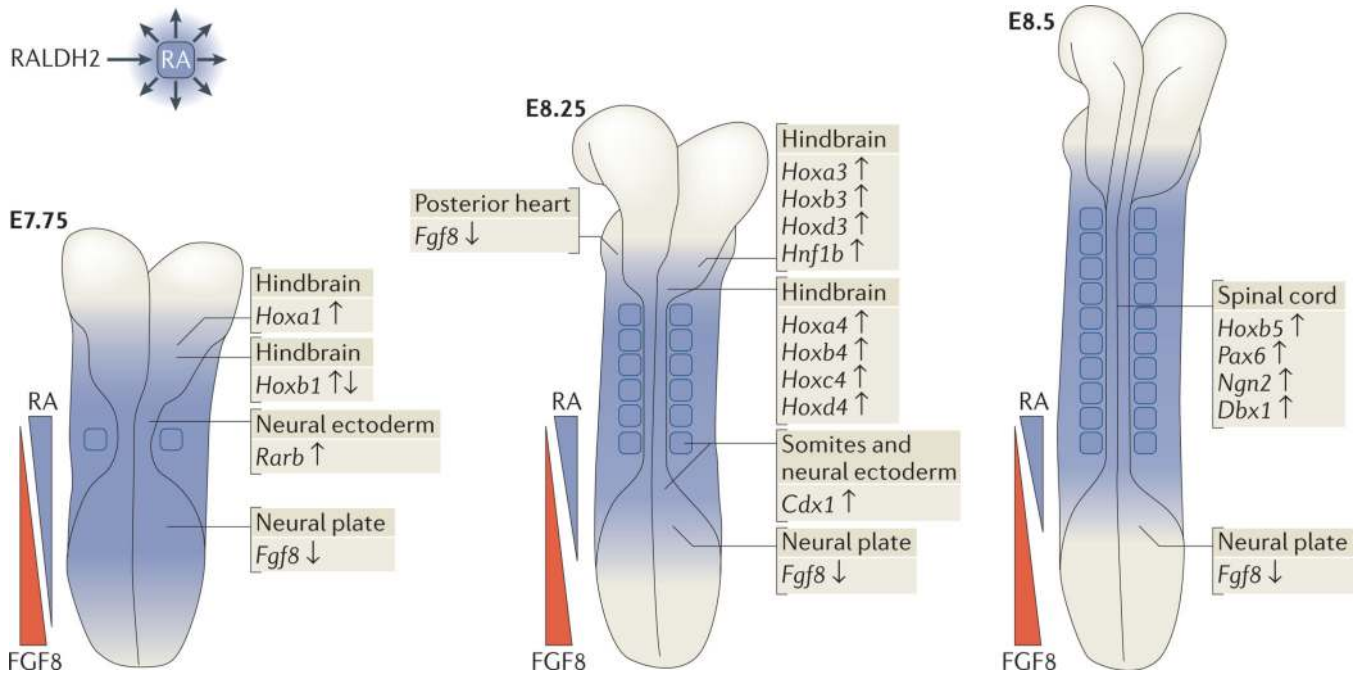


Figure 4. Direct RA target genes that are required for normal body axis extension, anteroposterior patterning, neurogenesis and somitogenesis

Retinoic acid (RA) generated by retinaldehyde dehydrogenase 2(RALDH2) in trunk mesoderm diffuses to nearby tissues to control several aspects of early mouse development. An overarching theme during these early stages is repression of caudal fibroblast growth factor 8 (*Fgf8*) by RA (depicted by opposing gradients of RA and FGF8) to allow normal body axis extension. In addition, RA activates homeobox (Hox) genes (and other genes) that are required for anteroposterior patterning of the trunk, neurogenesis and somitogenesis. Target genes are indicated with up-arrows for RA activation and down-arrows for RA repression. *Cdx1*, caudal-type homeobox 1; *Dbx1*, developing brain homeobox 1; *Hnf1b*, HNF1 homeobox B; *Ngn2*, neurogenin 2; *Pax6*, paired box 6; *Rarb*, retinoic acid receptor-β.

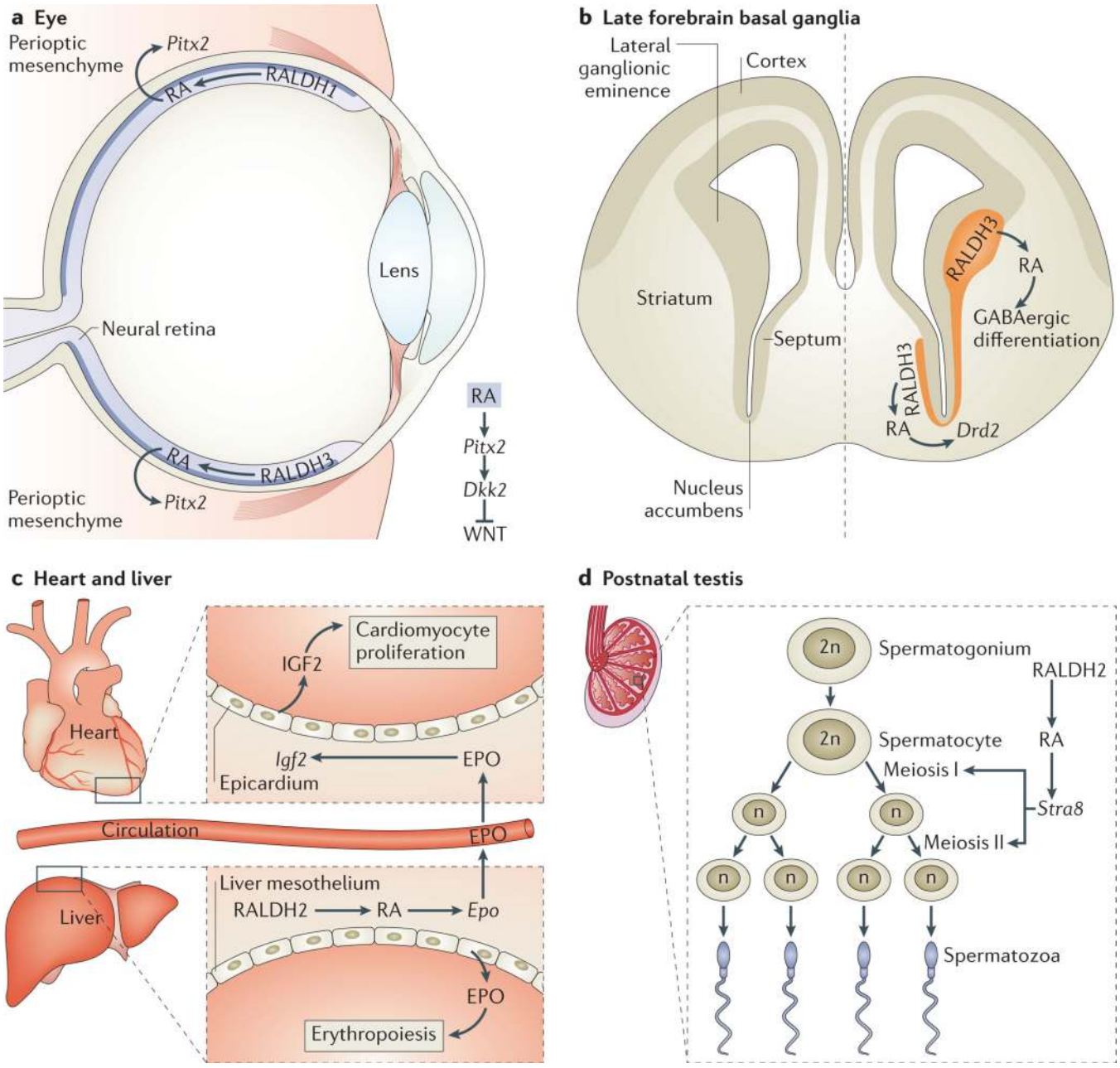


Figure 5. Diverse roles of RA in regulating mouse organogenesis

a | Eye: retinoic acid (RA) generated by retinaldehyde dehydrogenase 1 (RALDH1) and RALDH3 in the retina activates paired-like homeodomain transcription factor 2 (*Pitx2*) in perioptic mesenchyme, resulting in activation of dickkopf homologue 2 (*Dkk2*), which downregulates WNT signalling. **b |** In the late forebrain basal ganglia, RA generated by RALDH3 in the lateral ganglionic eminence and septum promotes γ-aminobutyric acid (GABA)-ergic differentiation by an unknown mechanism and stimulates dopamine signalling by activating dopamine receptor D2 (*Drd2*) in the nucleus accumbens within the striatum. **c |** In the heart and liver, RA generated by RALDH2 in the liver mesothelium activates erythropoietin (*Epo*) in fetal liver. *Epo* locally stimulates erythropoiesis and, via

transport of EPO in the circulatory system, stimulates myocardial proliferation through EPO-mediated upregulation of insulin-like growth factor 2 (*Igf2*) in epicardium. **d** | In the postnatal testis, RA generated by RALDH2 in spermatocytes activates *Stra8* (stimulated by retinoic acid gene 8) for the induction of meiosis.

Table 1

RAREs near genes known to require RA for normal expression in embryos

Gene	Function during development	Modality	Type	RARE sequence 5′ –3′ consensus[35]:			Refs
				AGGTCA G T	N?	AGGTCA G T	
Cdx1-5′	Somitogenesis and neurogenesis	Activating	DR2*	GGGTCG	TG	ACCCCT‡	110
		Activating	IR0*	GGGTCG		TGACCC	–
Cdx1-3′	Somitogenesis and neurogenesis	Activating	DR2	GGGTCA	AG	AGTTCA	111
Cyp26a1	Degradation of excess RA	Activating	DR5	AGTTCA	CCCAA	AGTTCA	97
Dbx1	Spinal cord interneuron development	Activating	DR2	TGTTCA	GC	TATTCA‡	123,162
Drd2	Forebrain striatum development	Activating	DR3	GGGTCA	CCC	TGGCCA	131,132
Epo	Liver erythropoiesis and cardiac growth	Activating	DR2	GGGTCA	AG	AGGTCA	138
Fgf8	Body axis extension, somitogenesis and forelimb initiation	Repressive	DR2	GGGTCA	GC	AGTTCA‡	30
Hnf1b	Hindbrain development	Activating	DR5	GGGTCA	CATTG	TGGTCA‡	163
Hoxa1	Hindbrain development	Activating	DR5	GGTTCA	CCGAA	AGTTCA‡	164,165
Hoxb1-5′	Hindbrain development	Repressive	DR2	AGGGCA	AG	AGTTCA‡	31
Hoxb1-3′	Hindbrain development	Activating	DR2	AGGTAA	AA	AGGTCA‡	166
Hoxb1-3′	Foregut and hindbrain development	Activating	DR5	GGTTCA	TAGAG	AGTTCA‡	167
Hoxa3	Hindbrain development	Activating	DR5	GGTTCA	AGAAG	AGTTCA	115,168
Hoxb3	Hindbrain development	Activating	DR5	GGTTCA	AGAAG	AGTTCA	115,168
Hoxd3	Hindbrain development	Activating	DR5	GGTTCA	AGCAG	AGTTCA	168
Hoxa4-5′	Hindbrain, spinal cord, gut, lung and kidney development	Activating	DR5	AGGTGA	ACTTC	AGGTCA‡	115,169
Hoxa4-3′	Hindbrain and spinal cord development	Activating	DR5	AGTTCA	CCGAG	AGGACA	115,168
Hoxb4-5′	Hindbrain and spinal cord development	Activating	DR5	GGGTGA	ACCGC	AGGTCA	170
Hoxb4-3′	Hindbrain and spinal cord development	Activating	DR5	AGTTCA	TGGAG	AGGCCA‡	115,170,171
Hoxc4-5′	Hindbrain and spinal cord development	Activating	DR5	AGGTGA	AATGC	AGGTCA	168
Hoxc4-3′	Hindbrain and spinal cord development	Activating	DR5	GGTTCA	CGGGA	AGGACA	168

Gene	Function during development	Modality	Type	RARE sequence 5′ –3′ consensus[35]:			Refs
				AGGTCA G T	N?	AGGTCA G T	
Hoxd4-5′	Anterior somite development	Activating	DR5	AGGTGA	AATGC	AGGTCA‡	172
Hoxd4-3′	Hindbrain and spinal cord development	Activating	DR5	GGTTCA	CCCAG	AGGACA‡	115,173
Hoxb5	Hindbrain and spinal cord development	Activating	DR5	GGATCA	CGCAG	AGGTCA‡	170,174
Mmp11	Limb interdigital development (two RAREs 200 bp apart)	Activating	DR2	AGGTCC	TG	AGTTCA	88,89,175
		Activating	DR2	AGGTCC	CG	AGTTCA	88,89,175
Ngn2	Spinal cord development (two RAREs 52 bp apart)	Activating	DR5	AGTTCA	CGCTA	TGGACA‡	122
		Activating	DR2	AGAACA	AA	AGCTCA‡	122
pou5f3	Maintainence of pluripotency	Repressive	DR2	CATTCA	CA	AATTCA‡	100
Pax6 Pitx2	Spinal cord and motor neuron development	Activating	DR2	AGTTCA	GT	TAGTCA	44,98,123
	Perioptic mesenchyme growth in the eye	Activating	DR5	AATTCA	TTAGA	AAGTCA	129
Rarb	RA signalling	Activating	DR5	GGTTCA	CCGAA	AGTTCA‡	176
Stra8	Male meiosis	Activating	DR2	GGGTGA	AA	AGGTCA	16,144
Tgm2	Limb interdigital development	Activating	DR5	AGGTCC	CAGTG	GGGTCA	88,177
Wnt8a	Body axis extension and somitogenesis	Repressive	DR2	AGATCA	GA	AGTTCA	119

All RARE sequences are mouse except *pou5f3*, which is from zebrafish. *Cdx1*, caudal-type homeobox 1; *Cyp26a1*, cytochrome P450 26A1; *Dbx1*, developing brain homeobox 1; DR2, direct repeat with 2-bp spacer; DR3, direct repeat with 3-bp spacer; DR5, direct repeat with 5-bp spacer; *Drd2*, dopamine receptor D2; *Epo*, erythropoietin; *Fgf8*, fibroblast growth factor 8; *Hnf1b*, HNF1 homeobox B; Hox, homeobox; IR0, inverted repeat with 0 spacer; *Mmp11*, matrix metalloproteinase 11; *Ngn2*, neurogenin 2; *Pax6*, paired box 6; *Pitx2*, paired-like homeodomain transcription factor 2; *pou5f3, POU domain, class 5, transcription factor 3*; RA, retinoic acid; *Rarb*, retinoic acid receptor-β; RARE, retinoic acid response element; *Stra8*, stimulated by retinoic acid gene 8; *Tgm2*, transglutaminase 2.

*The *Cdx1* upstream RARE was originally proposed to be an IR0, but we suggest that it could fit the DR2 type because 7 of 12 bases match with consensus direct repeat hexamers separated by 2 bp.

‡RARE function verified by mutational studies *in vivo*.