RNA regulons in Hox 5’ UTRs confer ribosome specificity to gene regulation

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Emerging evidence suggests that the ribosome has a regulatory function in directing how the genome is translated in time and space. However, how this regulation is encoded in the messenger RNA sequence remains largely unknown. Here we uncover unique RNA regulons embedded in homebox (Hox) 5’ untranslated regions (UTRs) that confer ribosome-mediated control of gene expression. These structured RNA elements, resembling viral internal ribosome entry sites (IRESs), are found in subsets of Hox mRNAs. They facilitate ribosome recruitment and require the ribosomal protein RPL38 for their activity. Despite numerous layers of Hox gene regulation, these IRES elements are essential for converting Hox transcripts into proteins to pattern the mammalian body plan. This specialized mode of IRES-dependent translation is enabled by an additional regulatory element that we term the translation inhibitory element (TIE), which blocks cap-dependent translation of transcripts. Together, these data uncover a new paradigm for ribosome-mediated control of gene expression and organismal development.

An important layer of post-transcriptional control of gene expression may be conferred through regulatory functions of the ribosome. For example, RPL38, one of 80 ribosomal proteins of the eukaryotic ribosome, helps establish the mammalian body plan by selectively facilitating the translation of subsets of Hox mRNAs, genes critically required for formation of the body plan. However, how ribosome-mediated regulation of gene expression is encoded within mRNA sequence remains an unanswered question.

All eukaryotic cellular mRNAs are capped, and cap-dependent translation is considered a highly efficient and predominant means for translation of most transcripts in the mammalian genome. In many viral mRNAs that are not capped, IRESs provide an alternative mechanism for ribosome recruitment to promote translation initiation. Interestingly, IRES elements have also been discovered in a handful of cellular mRNAs, including c-myc, XIAP, Apaf-1 and p53 (refs 11–16). As these cellular mRNAs are capped, their IRES elements act as a ‘fail-safe’ mechanism to promote translation under stress conditions, such as apoptosis or hypoxia, when cap-dependent translation is downregulated. Cellular IRES elements have little sequence or structural homology, and their relevance for translational control and gene expression in normal cell physiology, tissue patterning, or organismal development is unclear.

We report below the unexpected identification of IRES elements within Hox mRNAs, particularly those regulated by RPL38, which are critically required for accurate gene expression during normal development. We also identify the TIE, an additional regulatory element in these mRNAs, which modulates the dependence of individual transcripts on canonical cap-dependent translation. This mechanism enables the IRES to become the predominant mode of translation initiation and confers greater gene regulatory potential by the ribosome.

RPL38-regulated Hox mRNAs possess IRES elements

To address the nature of specific cis-regulatory elements within transcripts that confer greater regulation by the ribosome, we studied RPL38-mediated control of Hox mRNA translation. We showed previously that although RPL38 haploinsufficiency within the developing mouse embryo does not affect general cap-dependent translation, the translation of a subset of the eleven HoxA mRNAs (Hoxa4, Hoxa5, Hoxa9, Hoxa11) is perturbed (Fig. 1a). We employed a bicistronic reporter assay in the murine mesenchymal stem cell line C3H10T1/2, which expresses Hox transcripts, to delineate whether Hox 5’ UTRs direct cap-independent translation (Fig. 1a, b). In this reporter system, the first cistron (Renilla luciferase, RLuc) is translated by a cap-dependent mechanism, and the second cistron (Firefly luciferase, FLuc) is translated only if the preceding 5’ UTR element can recruit ribosomes by a cap-independent mechanism (Fig. 1a); the latter is considered to reflect IRES-dependent translation initiation.

These experiments revealed that many Hox 5’ UTRs possess IRES activity that is as strong as, or even stronger than, the hepatitis C virus (HCV) IRES element, a bona fide viral IRES element (Fig. 1b). To ensure that Hox 5’ UTRs do not contain putative promoters or splice sites that may produce a monocistronic capped FLuc transcript, we performed additional controls. As expected for a single transcript containing both RLuc and FLuc: (i) the RNA levels of the two reporters are expressed in the same ratio among all bicistronic constructs; (ii) a short hairpin RNA (shRNA) against RLuc downregulated both RLuc and FLuc expression; (iii) these transcripts do not contain cryptic splice sites; and (iv) no ribosome read-through of the first reporter is evident (Extended Data Fig. 1). There is good correlation between RPL38 regulation and the presence of IRES elements; all RPL38-regulated mRNAs in the HoxA locus possess IRES elements. These include Hoxa4, which we show by 5’ rapid amplification of cloned ends (RACE) possesses an IRES-containing 5’ UTR of 1.1 kb, which is longer than previously annotated.

To test whether cap-independent translation from the Hox 5’ UTRs is controlled by RPL38 in C3H10T1/2 cells, a copy of Rpl38 was disrupted by transcription activator-like effector nucleases (TALENs), yielding a 40% reduction in RPL38 protein expression (Fig. 1c, Extended Data Fig. 2a). In these cells, although there is no change in cap-dependent translation, there is a specific decrease in IRES-dependent translation of Hox target mRNAs that are regulated by RPL38 in vivo (Fig. 1c, 1 January 2015 | Vol 517 | Nature | 33

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Data represent mean ± s.d. (Fig. 1d, e). This region of the Hoxa9 5′ UTR shows remarkable evolutionary conservation in all vertebrates (from fish to mammals) that is greater than sequence conservation in the remaining 5′ UTR or the 3′ UTR sequence (Fig. 1f, Extended Data Fig. 3). IRES function is also evolutionarily conserved, as the zebrafish hoxa9b 5′ UTR shows strong IRES activity in murine C3H10T1/2 cells (Fig. 1g). These findings suggest that the Hoxa9 IRES element may have arisen early during vertebrate evolution for post-transcriptional regulation of Hox expression.

**Hoxa9 IRES is an RNA structure that recruits ribosomes**

Many viral IRESs possess structures such as conserved helices, asymmetric bulges, and pseudoknots that interact with initiation factors or the ribosome to promote translation initiation22–24. We therefore examined whether the Hoxa9 IRES element has structural properties in vitro that are functionally important.

Within the full-length Hoxa9 5′ UTR, protections of sub-regions of the 944–1,266 domain from selective 2′-hydroxyl acylation analysed by primer extension (SHAPE)25 were consistent with the minimal IRES domain forming a specific RNA structure in vitro (Extended Data Fig. 4). Automated modelling indicated that this domain formed a four-way junction with two long hairpin arms (P3 and P4; Fig. 2a, Extended Data Fig. 6) and a ‘right-angle’ asymmetric bulge (between P3b and P3c), but with little interaction with other domains of the 5′ UTR (Extended Data Fig. 4). Analogous chemical mapping and functional studies on the 5′ UTR from a different Hox mRNA, Hoxa5, also revealed a structured subdomain (nt 77–132) necessary for its full IRES activity (Extended Data Fig. 5a, d, e). Deletion of the subdomain substantially reduces IRES activity (Extended Data Fig. 5b). Structural conservation between different cellular IRES elements has so far been poorly defined19. Interestingly, the ‘right-angle’ asymmetric bulges observed in Hoxa5 and Hoxa9 (Extended Data Fig. 5c) bear qualitative similarity to asymmetric bulges in, for example, the HCV IRES26, suggesting at least certain common structural features are present amongst cellular Hox IRESs and viral IRESs.

To gain further insight into structural and functional properties of a Hox IRES, we applied multidimensional chemical mapping to the Hoxa9 minimal IRES subdomain (Fig. 2a, Extended Data Fig. 6). Comprehensive mutate-and-map analysis27 gave strong independent evidence for the same structural topology seen in the context of the full-length 5′ UTR, revealing exposure of RNA segments upon mutation of their predicted pairing partners (Extended Data Fig. 6a–c). Importantly, compensatory mutation analysis28 unambiguously confirmed specific base pairs in the P3 and P4 segments (green circles, Fig. 2a, and Extended Data Fig. 7). After this in vitro Hoxa9 structural characterization, we tested the functional significance of these hairpins for IRES-dependent translation through bicistronic assays. Deletion of P3, P4, or both hairpins in the context of the full-length Hoxa9 5′ UTR resulted in a significant reduction in IRES activity. Most strikingly, the mutation of nucleotides in the base of P3a reduced IRES activity, whereas compensatory mutations gave quantitative rescue of IRES activity (Fig. 2b).

These results demonstrate the importance of the P3 and P4 hairpins in IRES function and connect the detailed in vitro Hoxa9 IRES structural model to functional activity. Many viral IRES elements contain pseudoknots that may position the ribosome at the initiation codon29. A potential pseudoknot was suggested by mutate-and-map analysis (Fig. 2a), but this awaits further analysis. Together, these data confirm a structure defined at base-pair resolution for a Hox IRES element and indicate that a specific RNA structure is important for cellular Hox IRES activity.

To date, only a small class of viral IRES elements have been shown to interact with both the large and small ribosome subunits to form a translationally competent 80S ribosome30,31. However, a biotinylated full-length Hoxa9 5′ UTR, as well as the minimal IRES element contained within nt 944–1,266, are able to pull down ribosomal proteins from both the large and small subunits, including RPL38 (Fig. 2c, d). The full-length 5′ UTR also pulls down both 28S and 18S rRNAs (Fig. 2e), suggesting that the 80S ribosome is able to form on the
element from a Hoxa9 5' UTR monocistronic reporter construct dramatically decreases translational efficiency by 70-fold, despite the fact that the mRNA remains capped (Fig. 2f). We therefore hypothesized that the Hoxa9 5' UTR may contain additional element(s) that intrinsically inhibit cap-dependent translation. The Hoxa9 5' UTR contains 14 putative upstream open reading frames (uORFs), which are generally thought to inhibit the translation of the main open reading frame5. However, mutating all 14 ATGs to TTGs in the Hoxa9 5' UTR (AAATRESATuORF) (Extended Data Fig. 8a) does not affect cap-dependent translation of Hoxa9. We therefore placed various regions of the Hoxa9 5' UTR upstream of a monocistronic reporter to assay for effects on cap-dependent translation. Strikingly, nt 1–342 of the Hoxa9 5' UTR strongly inhibit cap-dependent translation (Fig. 2g) despite this region not being particularly GC-rich (55%) and therefore unlikely to impede ribosome scanning. Other similarly sized pieces of the 5' UTR (nt 343–643), or the very long remaining portion of the 5' UTR (nt 343–1,266), do not inhibit cap-dependent translation and are probably translated by a combination of cap and IRES-mediated translation. To our knowledge, the 1–342 fragment of the Hoxa9 5' UTR is a unique example of a 5' UTR regulon that is sufficient to strongly inhibit cap-dependent translation under physiological conditions. We termed this 5' UTR mRNA element the translation inhibitory element (TIE). SHAPE analysis of the full-length Hoxa9 5' UTR lacks evidence for structural interactions between the TIE and the minimal IRES (Extended Data Fig. 4a), suggesting they function as independent modules. These findings reveal that the TIE enables a more specialized mode of translation initiation directed by the IRES.

5' UTR topology allows for specialized translation

We next asked whether the unique 5' UTR topology of both a TIE close to the 5' end of the mRNA and an IRES element is a more generalizable mechanism of translational regulation. Our findings reveal that all the minimal IRES domains of HoxA 5' UTRs lie within the 100–300 nt closest to the AUG, with the exception of Hoxa5 (Fig. 3). Deletions of these minimal IRES domains in monocistronic reporter constructs reveal that they are critically required for normal translation (Fig. 4a–c). In addition, within these HoxA 5' UTRs, a TIE is also present near the mRNA cap (Fig. 4a–c). Moreover, although the Hoxa3, Hoxa4, Hoxa9 and Hoxa11 TIEs do not share obvious sequence similarity, they all strikingly block translation from the β-globin (Hbb) 5' UTR (Fig. 4d), which initiates translation exclusively by a cap-dependent mechanism13. Hoxa5 is the only 5' UTR that does not appear to possess a TIE and a putative uORF also does not decrease cap-dependent translation (Extended Data Fig. 8b). However, Hoxa5 has the shortest 5' UTR (237 nt) and almost the entire UTR is required for IRES activity, leaving little space for an additional TIE (Fig. 3c). Interestingly, the Hoxa5 transcript can be produced as a bicistronic mRNA with the neighbouring Hoxa6 gene37 and therefore may not need a TIE to inhibit cap-dependent translation in this context. These findings reveal a unique topology to Hox 5' UTRs that suppresses a more generic mode of cap-dependent translational control and facilitates specialized, ribosome-mediated control of protein expression.

Hoxa9 IRES is essential for translation in vivo

To determine whether IRES-dependent translation is critically required for the conversion of the Hoxa9 transcript into protein in vivo, we generated the first targeted mouse knockout of a cellular IRES by removing nt 944–1,193 of the Hoxa9 5' UTR, leaving the Kozak sequence and the rest of the 5' UTR intact (Fig. 5a, Extended Data Fig. 8c, d). A caveat to the previously generated Hoxa9 targeted knockout is the retention of a neomycin (Neo) cassette35,36, which can have indirect effects on the expression of neighbouring Hox genes37. Indeed, our current genetic studies reveal that the presence of the Neo cassette within the Hoxa9 targeting locus is sufficient to produce phenotypes previously attributed to Hoxa9 loss-of-function (Extended Data Fig. 9).
Therefore, in our Hoxa9<sup>ARES/ARES</sup> mouse, we were careful to remove the Neo cassette from the targeted locus by Cre-mediated excision.

Hoxa9<sup>ARES/ARES</sup> embryos do not show any major change in Hoxa9 transcript boundaries or expression levels (Fig. 5b, c). Strikingly, however, these mice show a fully penetrant homeotic transformation of the thirteenth thoracic vertebra (T13) to the first lumbar vertebra (L1), in which a full set of ribs normally present on T13 is missing (Fig. 5d). Although we cannot at present ascertain whether the Hoxa9 IRES deletion completely phenocopies a Hox9 knockout mouse, this dramatic homeotic transformation is precisely at the anterior boundary of Hoxa9 expression at the thoracic to lumbar transition. This transformation is also evident in ~50% of Hoxa9<sup>ARES/+ </sup> mice, although small rudimentary ribs can still form on T13. Consistently, there is a striking decrease in HOXA9 protein levels in the neural tube and somites of Hoxa9<sup>ARES/ARES</sup> embryos from the thoracic to sacral level (Fig. 6a) revealing a block in HOXA9 protein production. Furthermore, in Hoxa9<sup>ARES/ARES</sup> embryos there is a dramatic decrease in ribosome association of the Hoxa9 transcript. The majority of the Hoxa9 transcript in Hoxa9<sup>ARES/ARES</sup> embryos, but not control transcripts such as β-actin, accumulates in pre-polysomal fractions, reflecting an accumulation of mRNA not bound by translationally active ribosomal subunits (Fig. 6b, Extended Data Fig. 10). These findings reveal that the Hoxa9 IRES element is crucial in recruiting the ribosome to this transcript to pattern the mammalian body plan in vivo.

**Discussion**

Our studies reveal how expression information encoded within the mRNA template is sufficient to confer greater gene regulatory potential by the ribosome. In particular, our findings suggest that IRES elements are key regulatory elements within the mammalian genome that facilitate important, ribosome-mediated control of gene expression in development (Fig. 6c). Cellular IRES elements have been controversial because of their weak IRES activity as compared to viral IRESs.

Therefore, we have identified a common TIE that blocks cap-dependent translation of Hoxa9 IRES-mRNAs. a–c, Deletion of the minimal IRES elements within monocistronic reporters of Hoxa9 5′ UTR and identification of TIEs close to the mRNA cap in Hoxa9 (a), Hoxa4 (b) and Hoxa5 (c). All values are normalized to full-length constructs (FL); n = 3. d, Hoxa4 TIEs were placed upstream of the β-globin 5′ UTR (Hbb) in a monocistronic reporter to assay inhibition of cap-dependent translation, n = 3. All experiments were performed in duplicate. Data represent mean ± s.d. **P < 0.01 (t-test).
It will be interesting to determine if additional ribosomal proteins may promote specialized translation through control of unique subsets of IRES-containing mRNAs, either directly or through RNA-binding proteins. For example, RPS25 is required for IRES-dependent translation of certain viral IRES mRNAs. Moreover, RNA modifications both at the level of pseudouridylation and RPL13a-dependent methylation also appear to regulate the translation of certain cellular IRES-containing mRNAs. We therefore speculate that similar to the complex and highly regulated system of transcriptional control, in which specific DNA sequences and histone marks regulate gene expression, cis-acting RNA regulons, in conjunction with more specialized ribosome activity, provide newfound regulatory control to gene expression critical for mammalian development.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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IRES elements under non-stress conditions. Their relevance to normal gene regulation is also poorly understood, as all eukaryotic mRNAs are capped and undergo cap-dependent translation. Importantly, the requirement for IRES elements is explained by our identification of the TIE within these transcripts, which strongly inhibits cap-dependent translation. We speculate that such IRES elements may be more widespread in the genome and suggest a two-state mechanism for gene regulation, controlled by transcription and an additional layer of cis-acting RNA regulons within 5′UTRs that direct protein production. Both IRES and TIE elements may be differentially regulated, providing a versatile toolbox for controlling the ultimate expression of transcripts. Although IRES elements are highly conserved, and may already be present in Drosophila (14), the TIE lacks conservation and is absent even in vertebrate species such as fish and amphibians (15). These species often lay their eggs in harsh environments and may have originally used IRES elements as a fail-safe mechanism to ensure proper expression of key transcripts under stress conditions. Therefore, the acquisition of a TIE later in evolution may have enabled an additional, independent level of regulatory control to IRES-dependent translation and gene expression during normal development. A mechanism that regulates whether a pre-existing transcript is converted into a protein offers a fast and dynamic means to provide greater variation and control to gene expression.

Figure 6 The Hox9 IRES is critically required for Hox9 translation in vivo. a, Representative immunostaining of HOXA9 (green) in cross-section of E11.5 Hox9+/− (top) and Hox9−/− (bottom) embryos from the thoracic to sacral levels. DAPI (4′,6-diamidino-2-phenylindole) staining is in blue. NT, neural tube; som, somite. n = 3 embryos of each genotype. b, Somites and neural tubes of E11.5 embryos were microdissected and fractionated on a sucrose gradient. Middle, qPCR of Hox9 mRNAs in each fraction. Fractions 1–8 are pre-polysome fractions. **p < 0.01 (t-test compared to Hox9+/−). n = 3 embryos of each genotype. c, Model for how ribosome-mediated regulation of gene expression is encoded within the mRNA sequence. Stress-responsive cellular IRES elements are only active when cap-dependent translation is downregulated through a decrease in eIF4F activity. Hox9 mRNAs possess a TIE, which normally inhibits cap-dependent translation. The TIE enables ribosome-mediated control of Hox9 IRES expression during embryonic development.
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Author Information Hoxa4 5′ UTR sequence has been deposited in GenBank under accession number KM596709. All chemical mapping datasets have been deposited at the RNA Mapping Database (http://rmdb.stanford.edu) under the following accession codes: (1) Full-length: HOX4A5_STD_0000, HOX4A9_STD_0000; (2) Hoxa9 TIE: HOX4A9A_STD_0001; (3) Hoxa9 IRES: HOX4A9D_STD_0001, HOX4A9D_STD_0002, HOX4A9D_1M7_0001, and HOX4A9D_RSQ_0001. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.B. (mbarna@stanford.edu).
HindIII and NcoI sites. A primer containing the T7 promoter and a 3′ Fluc activity was normalized to total luciferase mRNA expression levels. Fluc primers to quantify total transcript abundance, the rest for luciferase assays. Cells were collected 24 h later. Then 50% of the cells were used for qPCR analysis with luciferase assays, various pGL3 constructs were transfected as above into 12-well plates. Cells harvested 72 h post-transfection for luciferase assays. For monocistronic luciferase Rluc or scrambled plasmid16 was cotransfected with the pRF constructs. Cells were harvested 24 h after transfection. Positive clones were identified by PCR followed by restriction digest. Rluc. To verify the integrity of the bicistronic construct, an shRNA plasmid against Rpl38 disruption was confirmed by Sanger sequencing and western blotting. Rpl38 transfection. Positive clones were identified by PCR followed by restriction digest. Rluc. To verify the integrity of the bicistronic construct, an shRNA plasmid against

5′ RACE. RNA was dissected from dissected E11.5 neural tubes and somites using TRizol (Invitrogen). The reverse transcription template was produced using ExactStart Eukaryotic mRNA 5′-3′ RACE Kit (Epitentre) and reverse transcribed using Superscript III (Invitrogen) with a primer in the coding region of Hoxa5. The 5′ UTR fragment was amplified by using Phusion High-Fidelity DNA polymerase (Thermo Scientific) with a gene specific reverse primer and a forward primer at the 5′ linker. PCR products were denatured, gel-extracted and subcloned into TOPO TA cloning vector (Invitrogen) for sequencing. TALEN. 2 sets of TALEN pairs were designed against the second and the third Hoxa4. The 5′ UTR fragment was amplified by using Phusion High-Fidelity DNA polymerase (Thermo Scientific) with a gene specific reverse primer and a forward primer at the 5′ linker. PCR products were denatured, gel-extracted and subcloned into TOPO TA cloning vector (Invitrogen) for sequencing. Luciferase assay. CH101T/1/2 cells were transfected in 24-well plates with various pRF constructs using Lipofectamine 2000 (Invitrogen) as per manufacturer instructions. Cells were collected 24 h post-transfection and assayed using Dual luciferase kit (Promega). IRES activity is expressed as a ratio between Fluc and Rluc. To verify the integrity of the bicistronic construct, an shRNA plasmid against Rluc or scrambled plasmid16 was cotransfected with the pRF constructs. Cells were harvested 72 h post-transfection for luciferase assays. For monocistronic luciferase assays, various pGL3 constructs were transfected as above into 12-well plates. Cells were collected 24 h later. Then 50% of the cells were used for qPCR analysis with Fluc primers to quantify total transcript abundance, the rest for luciferase assays. Fluc activity was normalized to total luciferase mRNA expression levels. **RNA pull down.** Template DNA was made by PCR from pRF-HoxA9 using a 5′ primer containing the T7 promoter and a 3′ primer containing a linker sequence T2 (Supplementary Table 1). Biotinylated RNA was in vitro transcribed with Megascript T7 kit (Ambion) using a final concentration of 6.8 mM each of ATP, CTP and GTP, 6.5 mM UTP and 0.3 mM Biotin-16-UTP (Roche). Synchronized RNAs were treated with TURBO DNase (Ambion) and purified by phenol/chloroform extraction. Unincorporated nucleotides were removed by running the RNA through a G50 column (GE Healthcare). The RNA was analysed by electrophoresis for quality and Nanodrop for quantity. A total of four 15-cm dishes of subconfluent CH101T/1/2 cells were collected for analysis by trypsinization. Cell pellets were washed and lysed on ice with 1.5 ml lysis buffer (10 mM Tris pH 7.5, 10 mM MgCl2, 150 mM NaCl, 0.1% TritonX, 0.25% NP40, 10 mM DTT, RNaseOUT, Halt Protease inhibitor) for 30 min. Lysate was centrifuged at 14,000 rpm for 10 min, 4°C. Supernatant was transferred to a new tube and cleared with 400 µl ahdin-agarose beads (Thermo) for 50 min, 4°C. Cleared lysate was transferred to new tube and quantified by spectrometry. Folding buffer was added to 100 pmol of biotinylated RNA of each species to a final concentration of 100 mM Tris pH 8, 100 mM NaCl, 10 mM MgCl2. RNA was heated to 95°C for 1.5 min, ice for 2 min and 37°C for 20 min. Then 100 pmol of folded RNA and 1 mg cleared lysate were incubated together with 100 µg ml−1 heparin and 100 µg ml−1 yeast RNA in 2 ml lysis buffer for 1 h at room temperature. Streptavidin M280 Dynabeads (Invitrogen) were washed according to manu-

factor’s instructions. 100 µl beads were incubated with each RNA per lysate sample for 1.5 h at 4°C. Beads were then washed with lysis buffer with increasing concentrations of salt up to 600 mM NaCl. One-tenth of the bead sample was kept for RNA analysis. The remaining nine-tenths were used for protein analysis. RNA was eluted by incubation for 5 min at 95°C. Eluted RNA was purified by phenol/chloroform extraction. RNA was converted to cDNA and analysed by qPCR using primers Hoxa95′ UTR F and Hoxa95′ UTR R (Supplementary Table 1). Proteins were eluted with 40 µl protein elution buffer (5 mM biotin, 3× SDS sample buffer) at 95°C for 5 min. Proteins were analysed by western blotting. **Western blotting.** Western blots were performed with antibodies against RP56 (Cell Signaling no. 2217) and RPL10a (Santa Cruz sc-100827). Polyclonal RPL38 antibodies were generated against C-EKAELKQLSPGGLAVDKLKR.

**RNA structural probing and data analysis.** Double-stranded DNA templates were prepared by PCR assembly of DNA oligomers with maximum length of 60 nt ordered from IBT (Integrated DNA Technologies). DNA templates for mutate-and-map (AM) and mutation/rescue were designed as previously described using NA_Thermo (https://github.com/DasLab/NA_thermo) and HiTRACE27,29. DNA template and RNA transcript preparation and quality checks were carried out as previously described27,28. All chemical mapping were carried out in 96-well format as described previously27,28,46. Briefly, before chemical modification, RNA was heated to 90°C for 30 seconds, cooled on the bench top to room temperature to remove secondary structure heterogeneity, and folded for 20 min at 37°C in 10 mM MgCl2, 50 mM Na-HEPES, pH 8.0. RNA was modified by adding 1/4 volume freshly made 5 mM 1-mercapto-7-nitroisatoic anhydride (1M7) in anhydrous DMSO or neat anhydrous DMSO as control. Modification reactions were incubated at room temperature and then quenched with 0.5 M Na-MES, pH 6.0. Quenches also included poly(dT) magnetic beads (Ambion) and FAM-labelled Tail2-A20 primer for reverse transcription. Samples were separated and purified using magnetic stands, washed with 70% ethanol twice, and air-dried. Beads were resuspended in ddH2O and reverse transcription mix, then incubated at 55°C for 30 min. RNA was degraded by addition of 1 volume of 0.4 M NaOH and incubation at 90°C for 30 min, then cooled and neutralized with an additional volume of acid quench (prepared as 2 volumes of 5 M NaCl2, 2 volumes of 2 M HCl, and 3 M NaOAc pH 5.2). Fluorescent labelled cDNA was recovered by magnetic bead separation, rinsed twice with 70% ethanol and air-dried. The beads were resuspended in Hi-Dis formamide containing ROX-350 ladder (Applied Biosystems), then loaded on capillary electrophoresis sequencer (ABI3100).

For full-length Hox transcripts, an Illumina MiSeq-based ligSHAPE (ligation-based SHAPE) on long RNAs was carried out, using a protocol analogous to Modseq27. RNA was folded similarly and probed by SHAPE as above, then recovered by ethanol precipitation. To obtain 3′ ends for ligating primer-binding sites at unstructured regions, modified RNA was folded again and fragmented by 5 mM TcCl4 for 5 min (or no TcCl4 as control)47, quenched by 0.5 M Na-EDTA pH 8.0, then purified by RNA cleanup and concentration columns (Zymo Research). Fragmented RNA was prepared through 3′ end phosphate removal by treatment with T4 polynucleotide kinase (New England Biolabs), T4 RNA-ligase-based ligation of 5′-adenylated universal miRNA cloning linker (New England Biolabs) to the 3′ end, reverse transcription to cDNA using sequencing primers, and circLigase-based Illumina adapter ligation to the cDNA 3′ end as described47.

The HiTRACE 2.0 package was used to analyse CE data, available as MATLAB toolbox at (https://github.com/hitrace)31 and web server at (http://hitrace.org)32. Electroreticular traces were aligned and baseline subtracted using linear and non-linear alignment routines as previously described27. Band intensities were fitted to Gaussian peaks, then background subtracted, signal attenuation corrected and normalized. MiSeq results were analysed using the MASeqer software package30,34. The SHAPE reactivity profile was generated by projection of pair-ended reads. Briefly, a correction factor of 0.25 was used for full-length read ligation bias correction, accounting for the empirically observed low circLigase ligation efficiency for full-length cDNAs ending in CC35. Reads shorter than 8 nt were discarded due to ambiguity in their positional alignment in the full-length RNA. Modification fractions were determined by the number of cDNAs in which reverse transcription stopped at a given nucleotide divided by the number of cDNAs in which reverse transcription stopped or proceeded beyond that nucleotide36. Traces were background subtracted (SHAPE minus no-SHAPE data; and SHAPE-Tb minus Tb-alone data) and normalized through a boxplot-based heuristic45 for Hoxa5, or using a scanning window of 350 nt for Hoxa9. Errors were estimated as the standard deviation between SHAPE-no-SHAPE and SHAPE-Tb/Tb-alone measurements.

Data-guided secondary structure models were obtained using the Fold executable of the RNAstructure package37. Pseudo-energy parameters of RNAstructure version 5.4 were used by default. To obtain 2D-data-guided secondary structure models, Z score matrices for mutate-and-map data sets were calculated and used as base-pair-wise pseudo-energies with a slope and intercept of 1.0 kcal mol−1 and 0 kcal mol−1 as previously described27. The ShapeKnots executable of RNA structure version 5.6 (ref. 58) enables modelling of pseudoknots but with different parameters for pseudoknot modelling than SHAPE (Ref. 37). ShapeKnots was used for pseudoknot prediction of Hoxa9 (5′7-1,332 when using 1D and 2D SHAPE data combined. Extension of ShapeKnots to accept 2D data are being incorporated into the next version of RNAstructure. Helix-wise confidence values were calculated via bootstrapping as described previously27,28. All predictions were

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modelled in context with full-length sequences. Secondary structures were visualized by the VARNA software\(^1\).

**Mice.** A 2.8-kb 5′ arm and a 4.9-kb 3′ arm of Hoxa9 were cloned into PGKneolox2DTA.2 (P. Soriano, Addgene plasmid 13449). This results in a replacement of nt 945–1,194 of Hoxa9 5′ UTR with a LoxP-flanked Neo cassette in the same transcriptional direction as Hoxa9 (Extended Data Fig. 8c). The plasmid was linearized with XhoI and electroporated into J1M8N3A3.1 ES cells for homologous recombination by the UCSC ES cell core. The correctly targeted ESC clones were identified by long-range PCR and confirmed by Southern blotting. The clones were injected into C57BL/6 blastocysts by the Gladstone Institute Transgenic Core Facility. Resulting chimaeras were crossed with C57BL/6 mice to generate Fl (Hoxa9\(^{Neo^+}\)). Hoxa9\(^{Neo^+}\) were crossed with CMV-Cre mice (Jackson Laboratory) to precisely remove the LoxP-flanked Neo cassette within the 5′ UTR to generate Hoxa9\(^{RES+}\) mice. All mice used for experiments were between 2 and 6 months old. All animal studies were performed in accordance with Stanford University Animal Care and Use guidelines.

**Sucrose gradient fractionation.** E11.5 neural tubes and somites were dissected and trypsinized as previously described\(^1\). The neural tube and somites of each embryo were lysed on ice in 200 μl of lysis buffer (20 mM Tris pH 7.5, 200 mM NaCl, 15 mM MgCl\(_2\), 100 μM cycloheximide, 100 μg ml\(^{-1}\) heparin, 200 μU ml\(^{-1}\) SUPERase In (Ambion)). After lysis, nuclei and membrane debris was removed by centrifugation (1,300g, 5 min at 4 °C, and then 14,000 rpm, 5 min, at 4 °C). The supernatant was layered onto a linear sucrose gradient (10–40% sucrose(w/v), 20 mM Tris, pH 7.5, 100 mM NaCl, 15 mM MgCl\(_2\), 100 μg ml\(^{-1}\) cycloheximide) and centrifuged in a SW41Ti rotor (Beckman) for 2.5 h at 40,000 rpm at 4 °C. Fractions were collected by Density Gradient Fraction System (Brandel). For sucrose gradient fractions, amount of RNA from individual fractions was analysed and converted to relative RNA quantity using CFX manager (BioRad). cDNA was diluted twofold and 1 μl of cDNA used at 250 nM per reaction. Primers were previously described\(^1\) and used at 250 nM per reaction.

**qPCR analysis.** For cells, RNA was isolated using NucleoSpin RNA kit (Clontech) and DNase-treated on column. For embryos, E11.5 neural tubes and somites were dissected and trypsinized as previously described\(^1\). RNA was isolated using TRIzol (Invitrogen) and treated with TURBO DNA-free kit (Ambion). For sucrose gradient fractions, amount of RNA from individual fractions was analysed and converted to relative RNA quantity using CFX manager (BioRad) and qPCR assay (SsoAdvanced SYBR Green supermix and CFX384, BioRad). Data was analysed and converted to relative RNA quantity using CFX manager (BioRad). For sucrose gradient fractions, amount of RNA from individual fractions was expressed as a fraction of the total RNA collected from all fractions. Primers were used at 250 nM per reaction. β-actin and Hoxa9 qPCR primers were previously described\(^1\). Fluc F 5′-CATCACGTAGCGGGATACCTT, Fluc R 5′-AAGAGATA CGCCGGTGTTTC. 18S F 5′-ACATCCAAAGAAGCCACGAC, 18S R 5′-ATTCC CAAATTCAGGGGCTT, 28S F 5′-GGGGAGAGGGTGTAAATCTC, 28S R 5′- T CCTTATCCCGAATTACGG.

**Whole mount in situ hybridization.** Whole mount in situ hybridization was carried out using digoxigenin-labelled antisense RNA probes as previously described\(^1\). Hoxa9 probe was amplified from mouse embryo cDNA. Primers sequences are described in Supplementary Table 1.

**Skeletal staining.** Alcian blue and alizarin red staining of cartilage and bone were performed on neonates as previously described\(^1\).

**Immunofluorescence.** E11.5 embryos were dissected, fixed and cryosectioned as previously described\(^1\). WT and Hoxa9\(^{RES+}\) littermates sections were placed onto the same slide for direct comparison and identical processing. Sections were equilibrated to room temperature and rehydrated in blocking buffer (1% heat-inactivated goat serum, 0.1% Triton X-100 in PBS) for 1 h at room temperature. Sections were incubated with anti-HOXA9 antibody (Abcam ab140631) at 1:1,000 in blocking buffer overnight at 4 °C, washed three times, 30 min each and incubated with Alexa 488 anti-rabbit secondary antibodies (Invitrogen) for 1 h at room temperature. Slides were washed with blocking buffer three times, 30 min each and incubated with 1 μg ml\(^{-1}\) DAPI (Cell Signalling) for 15 min at room temperature. They were washed again in blocking buffer for 20 min and mounted with VectaShield Mounting Media (Vector Laboratories).

**Statistical analysis.** No normalization, blinding or exclusion was used in any experiments. No statistical method was used to predetermine sample size. In all cases, multiple independent experiments were performed on different days to verify the reproducibility of experimental findings. For mice experiments, embryos from multiple litters were used to avoid litter-specific bias.

Each variable was analysed using the two-tailed unpaired Student’s t test. For all analyses, a P value of less than 0.05 was considered significant. Results are shown as means ± s.d.

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Extended Data Figure 1 | HoxA IRES controls confirming that Fluc activity from bicistronic vector is due to IRES activity. a, qPCR of both Rluc and Fluc from transfected cells shows that Rluc and Fluc are produced at the same ratio. All Rluc and Fluc values are normalized to that of HCV (set to 1). $n = 3$, individual experiments performed in duplicate. b, c, shRNA against Rluc decreased reporter activity of both Rluc (a) and Fluc (b), confirming that Rluc and Fluc were transcribed on the same mRNA. $n = 3$, individual experiments performed in triplicate. d, RT-PCR using primers in Rluc and Fluc show that there is no cryptic splice site in the cloned Hox 5’ UTR. Primer locations are shown as arrows in the diagram. e, Inserting a strong hairpin ($-67\text{ kcal mol}^{-1}$) after the Rluc reporter did not affect Fluc activity, suggesting that Fluc activity was not due to ribosome read-through.
Extended Data Figure 2 | Disruption of Rpl38 in C3H10T1/2 by TALEN nucleases. **a**, Location of TALEN pairs. Two pairs of TALENs were designed to bind at the end of exon 2 and the beginning of exon 3 to make a genomic break close to the ATG. Sequencing of a positive clone shows a deletion of the ATG and most of the intron after it. Coding sequence is highlighted in green. **b**, Rpl38 knockdown does not change cap-dependent translation (Rluc) but decreases IRES-dependent translation (Fluc) from specific Hox 5′ UTRs. Luciferase activity was normalized to amount of Fluc RNA in the cells as quantified by qPCR. *P < 0.05 (t-test compared to control). n = 2, individual experiments performed in duplicate.
Extended Data Figure 3 | Alignment of the Hoxa9 IRES element between vertebrate species. Nucleotides 945 to 1,266 of the mouse Hoxa9 5' UTR were aligned with sequences from other vertebrates and show high sequence homology. Nucleotides are coloured based on their homology, with darker colours representing higher conservation.
Extended Data Figure 4 | Chemical mapping and secondary structure prediction of full-length Hoxa9 5' UTR. a, Secondary structure modelling of full-length Hoxa9 using ligSHAPE data. The Hoxa9 IRES element (nt 957–1,132, shaded in green) is modelled as the same secondary structure shown in Fig. 2a. Confidence values from bootstrapping (bulge percentages) exceed 90% for this element, suggesting a well-determined subdomain, but are generally low outside this region, indicating poor certainty in other regions. b, Normalized SHAPE reactivity of Hoxa9 IRES (nt 957–1,132 and 944–1,266 from one-dimensional SHAPE read out through capillary electrophoresis (CE), full-length 1–1,266 from MiSeq-based ligSHAPE). c, Normalized SHAPE reactivity of Hoxa9 TIE (nt 1–342 from CE-based one-dimensional SHAPE, full-length 1–1,266 from MiSeq-based ligSHAPE).
Extended Data Figure 5 | Chemical mapping and secondary structure model of full-length Hoxa5 5' UTR. a, Secondary structure modelling of Hoxa5 using one-dimensional SHAPE data. Nucleotides are coloured with SHAPE reactivities. Percentage labels give bootstrap support values for each helix. The feature highlighted in blue resembles P3 in Hoxa9 and the tip highlighted in pink is deleted in b. b, The deletion of the tip identified in Hoxa5 IRES structure shown in a decreases IRES activity in bicistronic reporter assays. IRES activity was normalized to full length Hoxa5 5' UTR (A5, set to 1). **p < 0.01 (t-test as compared to A5). n = 2 experiments, performed in triplicate. c, Both Hoxa9 and Hoxa5 contain an asymmetric bulge in a region important for IRES activity. d, e, Normalized SHAPE (d) and DMS (e) reactivity of Hoxa5 (CE-based and MiSeq-based).
Extended Data Figure 6 | Secondary structure model and mutate-and-map (M²) data set of Hoxa9 IRES element. a, b, Entire M² data set and Z-score contact-map of Hoxa9 nt 957–1,132 across 177 single mutants probed by 1M7. c, Secondary structure model of Hoxa9 nucleotides 957–1,132 using M² data alone. d, Secondary structure model of Hoxa9 nt 957–1,132 using one-dimensional SHAPE data alone. Nucleotides are coloured with SHAPE reactivity. e, Secondary structure model of Hoxa9 nt 944–1,266 using one-dimensional SHAPE data. The models in c–e contain the same helices as the model from combined SHAPE/M² analysis in Fig. 2a, up to register shifts and edge base pairs; the small rearrangements are labelled P3b’, P3c’, P3d, P4b’ and P4c’.
Extended Data Figure 7 | Mutation/rescue results of Hoxa9 IRES structure (nt 944–1,266) probed by 1M7. Electropherograms of mutation/rescue to test base-pairings in P3c (a, b), P3b (c–e), P3a (f–k), P4b (l–o), P4a (p–q), and pk3-4 (v–ai). Perturbation of the chemical mapping reactivities by mutations of one strand and restoration by mutations in the other strand provide strong evidence for the tested pairings in P3c (a, b), P3b (c–e), P3a (f–k), P4b (l–o), and P4a (p–q). Near-perfect restoration by compensatory mutations in (x) and (ad) support pseudoknot pk3-4. Lack of rescue in other tested pairings is consistent with either absence of those pairings or higher-order structure (for example, base triples) interacting with those pairings.
Extended Data Figure 8 | Putative uORFs within the 5’ UTRs of Hoxa9 and Hoxa5 do not inhibit cap-dependent translation and Hoxa9^IRES^ targeting strategy. uORFs are marked by black circles on the diagram of monocistronic reporter for the Hoxa9 (a) and Hoxa5 (b) 5’ UTR. All the ATGs in the 5’ UTR were mutated to TTG in A9^ΔuORF construct and GTG in A5^ΔuORF. The IRES element (944–1,266) was removed in A9^IRES construct. The IRES element was removed from the A9^ΔuORF construct in A9^ΔIRESΔuORF. n = 3 individual experiments in duplicates. Data represent mean ± s.d. c, Diagrams of the Hoxa9 locus and the targeting vector. Boxes represent exons, grey boxes represent UTRs, and black boxes represent the coding sequence. Nucleotides 944–1,145 were replaced by a floxed Neo cassette in the targeting vector. Locations of Southern blot probes, restriction enzymes used for Southern analysis and expected sizes are marked on the diagrams. d, Southern blot analysis of targeted cells using both the 5’ and 3’ probes showing that both arms integrated correctly into the Hoxa9 locus. Mice were generated from clone P3A5.
Extended Data Figure 9 | The presence of a Neo cassette in the Hoxa9 locus is linked to the presence of an L1 → T13 homeotic transformation.

a, Diagram of the Hoxa9 locus (top) and axial skeleton phenotype (bottom) in different Hoxa9 mouse mutants. The original Hoxa9<sup>−/−</sup> was made by replacing the homeodomain with a Neo cassette. Vertebra with homeotic transformation is coloured red. b, Representative skeletons of Hoxa9<sup>+/+</sup>, Hoxa9<sup>Neo/+</sup> and Hoxa9<sup>Neo/Neo</sup>. Arrows point to the additional rib(s) on L1, revealing a homeotic transformation to T13. These results show that it is the presence of Neo in the targeting locus, which may affect the expression of neighbouring Hox gene, that is sufficient to cause the L1 → T13 phenotype. When the Neo cassette is removed from the targeting locus by crossing the Hoxa9<sup>Neo/+</sup> mouse with a CMV Cre line, the L1 → T13 phenotype is no longer present. n = 3 skeletons of each genotype.
Extended Data Figure 10 | Sucrose gradient fractionation shows no difference in β-actin association with polysomes in *Hoxa9*+/+ and *Hoxa9*AIRES/AIRES embryos. a, Overlay of A260 trace during fractionation showing no difference in polysome profiles between E11.5 *Hoxa9*+/+ and *Hoxa9*AIRES/AIRES embryos. b, qPCR from each fraction reveals no difference in β-actin mRNA accumulation between *Hoxa9*+/+ and *Hoxa9*AIRES/AIRES embryos. c, Quantification of β-actin mRNA in fractions. Fractions 1–8 are pre-polysomes and 9–16 are polysome fractions. n = 3 embryos of each genotype.