Genomic Targets of Brachyury (T) in Differentiating Mouse Embryonic Stem Cells

Amanda L. Evans1,4, Tiago Faial2,3,4, Michael J. Gilchrist1,4, Thomas Down1, Ludovic Vallier2, Roger A. Pedersen2, Fiona C. Wardle5, James C. Smith1,3,4,6

1 Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Cambridge, United Kingdom, 2 The Anne McLaren Laboratory for Regenerative Medicine, University of Cambridge, Cambridge, United Kingdom, 3 Department of Zoology, University of Cambridge, Cambridge, United Kingdom, 4 Medical Research Council, National Institute for Medical Research, London, United Kingdom, 5 Randall Division of Cell and Molecular Biophysics, King’s College London, London, United Kingdom

Abstract

Background: The T-box transcription factor Brachyury (T) is essential for formation of the posterior mesoderm and the notochord in vertebrate embryos. Work in the frog and the zebrafish has identified some direct genomic targets of Brachyury, but little is known about Brachyury targets in the mouse.

Methodology/Principal Findings: Here we use chromatin immunoprecipitation and mouse promoter microarrays to identify targets of Brachyury in embryonic bodies formed from differentiating mouse ES cells. The targets we identify are enriched for sequence-specific DNA binding proteins and include components of signal transduction pathways that direct cell fate in the primitive streak and tailbud of the early embryo. Expression of some of these targets, such as Axin2, Fgf8 and Wnt3a, is down regulated in Brachyury mutant embryos and we demonstrate that they are also Brachyury targets in the human. Surprisingly, we do not observe enrichment of the canonical T-domain DNA binding sequence 5’TCAACCT-3’ in the vicinity of most Brachyury target genes. Rather, we have identified an (AC)n repeat sequence, which is conserved in the rat but not in human, zebrafish or Xenopus. We do not understand the significance of this sequence, but speculate that it enhances transcription factor binding in the regulatory regions of Brachyury target genes in rodents.

Conclusions/Significance: Our work identifies the genomic targets of a key regulator of mesoderm formation in the early mouse embryo, thereby providing insights into the Brachyury-driven genetic regulatory network and allowing us to compare the function of Brachyury in different species.

Introduction

Brachyury (T) is expressed in the primitive streak, tailbud and notochord of the early mouse embryo [1,2]. It plays a key role in early development: mouse embryos lacking functional Brachyury protein do not gastrulate properly, fail to form a differentiated notochord, lack structures posterior to somite seven, and have defects in left-right patterning [3,4,5]. The expression patterns of the Xenopus [6] and zebrafish [7,8] Brachyury orthologues resemble those of the mouse, and these genes play similar roles in early development [9,10,11], indicating that Brachyury function has been conserved throughout evolution.

In an effort to understand how Brachyury exerts its effects, we have searched for genomic targets of this transcription factor. In previous work using Xenopus embryos we have used differential screening approaches to isolate target genes such as eFGF [12], members of the Bix family [13,14] and Wnt11 [15], while a chromatin immunoprecipitation-microarray (ChIP-chip) approach in the zebrafish embryo has allowed us to identify more than 200 potential targets of Nota tail a (Nta), the orthologue of Brachyury [16]. In this paper, we apply a ChIP-chip approach to identify targets of Brachyury during mouse embryonic stem (ES) cell differentiation. ES cells provide an abundant source of material as they differentiate towards embryoid bodies (EBs), and we predict that the identification of Brachyury targets in these cells will shed light on ES cell differentiation as well as help identify such targets in the early embryo. This work might also indicate the extent to which the biological function of Brachyury...
has been conserved in vertebrates and provide information on how Brachyury binding motifs are disposed within target cis-regulatory regions.

Our results show that Brachyury targets in differentiating ES cells are enriched for sequence-specific DNA binding proteins and components of signal transduction pathways that direct cell fate in the primitive streak and tailbud of the early embryo. Interestingly, most binding peaks were not enriched for the canonical T-box binding site 5'-TCACACCT-3' [17,18] but did contain a repeating AC motif. Amongst the signal transduction pathway components were regulators of the WNT and FGF pathways. Our results show that Brachyury targets in differentiating ES cells are enriched for sequence-specific DNA binding proteins and components of signal transduction pathways that direct cell fate in the primitive streak and tailbud of the early embryo. Interestingly, most binding peaks were not enriched for the canonical T-box binding site 5'-TCACACCT-3' [17,18] but did contain a repeating AC motif. Amongst the signal transduction pathway components were regulators of the WNT and FGF pathways. These include Axin2 (Axil/Conductin), which encodes a negative regulator of Wnt signalling, as well as Wnt3a and Fgf8. Significantly, expression of all three genes is down regulated in homozygous Brachyury mutant embryos and we show by ChIP-qPCR that these are also genomic targets of BRACHYURY in differentiating human ES cells. These results are consistent with work in the zebrafish emphasising the importance of Wnt and Fgf signal transduction pathway components as Brachyury targets [16]. In demonstrating that expression of Axin2 in the early mouse embryo is regulated by Brachyury as well as by TCF/LeF proteins [19,20,21,22], our results emphasise the complex interplay between signalling pathways in the regulation of gene expression in the early embryo.

**Results**

**ES cell culture**

Preliminary experiments demonstrated that our ES cell culture regime yielded embryoid bodies of uniform size, similar to that of EBs grown on hydrophobic surfaces [23], and that expression of Brachyury usually peaked at day 4 of differentiation (Fig. 1). Immunohistochemical analysis indicated that approximately 15% of cells in our embryoid bodies express Brachyury (data not shown).

**ChIP-chip and bioinformatic analysis**

Binding sites of Brachyury were identified by ChIP-chip experiments and the closest gene was identified using version NBI35.1 of the annotated mouse genome (see Material and Methods). Following filtration, our analysis gave a list of 520 enriched probes representing 396 genes (Table S1). Genomic quantitative PCR on a selection of genes called as bound or unbound confirmed that our ChIP-chip approach identified genuine binding events (Fig. S1).

Brachyury is expressed at its highest levels in the primitive streak and haematopoietic progenitors at E7.5 to E8.5. Later expression is restricted to the tailbud and notochord (E12.5), and then to parts of the brain and tail [2]. Of the Brachyury targets identified in our embryoid body experiments whose expression patterns are known, most (63%) are activated during this period of 7.5 to 17.5 dpc of mouse development (Fig. 2A). And of these 250 genes, many are restricted to the primitive streak or its mesodermal derivatives, with 30% (75 transcripts) expressed exclusively in the mesoderm (Fig. 2A). In addition to Axin2, Wnt3a and Fgf8, which are discussed below, genes that have been reported to be co-expressed with Brachyury include Msgn1, whose expression is down regulated in Brachyury mutants [24], Meis1 [25] Trim28 [26] and Zic2 [27], which are expressed in the primitive streak during gastrulation, Foxa2, present in the node and notochord [28], and Adam19 (meltrin beta), present in tailbud mesenchyme [29]. These and other transcripts (see below) are also co-expressed with Brachyury (or are activated shortly after Brachyury) in embryoid bodies, and typical profiles of Msgn1, Meis1 and Foxa2 expression are shown in Fig. 2B.

The targets we identify include components of the WNT, MAPK, JNK, TGF-β, Hedgehog, FGF and G-protein coupled signal transduction pathways (Table S2). Analysis of the targets yielded a set of gene ontology (GO) terms consistent with the function of Brachyury during gastrulation [30]. In particular, cellular component analysis highlights gene products involved in morphogenesis, cell adhesion and cell

**Figure 1. Temporal expression pattern of Brachyury during early ES cell differentiation.** The graph shows a quantitative RT-PCR profile from an embryoid body spinner culture. Brachyury expression is calculated relative to beta actin. Images show undifferentiated R1 cells on mouse embryo fibroblast feeders at day 0, early blast colonies at day 2, and embryoid bodies at days 3, 4 (when they are cross-linked) and 5.

doi:10.1371/journal.pone.0033346.g001
polarity. Targets such as Gdf5, Lmx1b and Dlx5 are involved in morphogenesis (Fig. S2A: GO:0048598; P<10^-5), Gabral, Gfna3 and Cbhn3 are involved in anchoring the plasma membrane to cytoskeletal proteins (Fig. S2B: GO:0030054; P=1×10^-7) and others encode proteins involved in cell adhesion, such as the glycosyltransferases B4galt2 and Cnfl//Nrtl. This last observation is consistent with data showing that glycosyltransferases, and especially galactotransferases, are mis-expressed in T/T mutant mice [31], that the extracellular matrix is reduced in such embryos [32], and that cells have fewer cytoplasmic processes, especially in the somites and mesenchyme [32]. Significantly, over-expression of Cnl4, or its Xenopus orthologue Camellos (Xcm1), inhibits gastrulation in Xenopus [33].

Interestingly, another group of targets is associated with germ cells (Table S3), including Aplil1 (Ddefl), which encodes an ADP-ribosylation factor GTPase-activating protein implicated in metastatic prostate cancer [34], and also the Wilms' tumour gene WT1 [35].

Transcription factor targets

Of the 396 genes identified as potential targets of Brachyury, 53 (13.4%) are transcription factors (Table S4), and indeed gene ontology analysis demonstrates significant enrichment for sequence-specific DNA binding proteins (Fig. S2C: GO:0043565, p<10^-5). Several families of transcription factors are represented, including the Ets, paired box, homeobox, winged helix/forkhead, bZip and zinc finger families. Under our ES cell culture conditions, expression of many of these transcription factors peaks either at the same time as Brachyury (Foxa2, Foxe1) or just afterwards (Bapxi, Ebf2, Egr, Hoxa13, Mesi1, Msn1, Nkx2.6 and Slug) (Fig. 2B). As we discuss below, our data provide a basis for deciphering the transcription factor genetic regulatory network underlying mesoderm formation in ES cells and in the embryo.

T-box protein binding motifs

Previous work indicates that Brachyury interacts with the sequence 5'-TCACACCT-3' [16,36,37,38]. To our surprise, neither nested MICA nor RSAT identified this motif as significantly enriched in the DNA sequences selected in our experiments. Rather, both packages identified enrichment of the simple sequence repeat (AC)n (Fig. S3A). However, although it was not enriched, we did observe that several regulatory regions contain a sequence resembling a T-box site (in which 1 to 3 nucleotides differ from the consensus) close to an AC repeat. These genes include Axin2, Camil1/β-catenin, Egr, Ekb1, Egfl8, Foe, Foxa2, Foxe1, Fyb, Id4, Mesi1, and Hoxa3 where the T-box-like sites may be positioned either 5' or 3' to the repeat sequence.

To assess the significance of these observations we first performed electrophoretic mobility shift assays (Fig. S3B). As expected, the T-domain of mouse Brachyury binds the canonical TCACACCT sequence, binding can be competed by unlabelled oligonucleotide, and the complex can be “super-shifted” by a Brachyury antibody. The (AC)n repeat motif also forms a complex with Brachyury, but although the complex can also be ‘supershifted’, unlabelled oligonucleotide competes very poorly. Finally, when both motifs are present in the radiolabelled oligonucleotide, competition using an excess of cold oligonucleotide in which just the T-box site is mutated is poor, and so is competition in which just the (AC)n region is mutated. Together, these observations indicate that just the (AC)n sequence interacts only weakly with Brachyury, if at all, and that its role may be restricted to stabilizing binding to an adjacent or even a distant T-box site.

If true, such a role is likely to be restricted to rodent species. Our dataset contains 111 peaks with associated AC repeats longer than eight nucleotides (Table S5). Comparison with rat, human, zebrafish and Xenopus genomes shows that 38 of these AC-rich regions are unique to the mouse while 68 are also present in the rat. Sixteen of the AC repeats are present in the human genome, of which 11 are also present in rat. However, none of the repeats are conserved in zebrafish or Xenopus (Table S5).

Axin2 and Wnt3a as targets of Brachyury

Amongst the identified Brachyury targets are many genes encoding positive and negative regulators of the Wnt signalling pathway (Fig. 3A). Enrichment peaks in the promoter regions of Dkk1, Cnfl//β-catenin, Dc33, and γ-catenin/Top show Brachyury binding (Fig. 3B, C, D and E) and also reveal the presence of AC repeats (green bars) and imperfect T-binding sites (blue bars). Of these Wnt-related genes, Wnt3a and Axin2 both show strong Brachyury binding peaks around their transcription start sites in our ChiP-chip analyses (Figs. 4A, 5A), and their temporal expression patterns both resemble that of Brachyury in our embryoid body system (Figs. 4B, 5B). For Wnt3a, a variant Brachyury site is positioned close to an AC repeat sequence in the first intron, and a canonical TCACACCT Brachyury site is upstream of the transcription start site (Fig. 4A). In the case of Axin2, a canonical Brachyury site is positioned close to a variant site and to an AC repeat (Fig. 5A).

To ask whether Brachyury is required for expression of Wnt3a and Axin2, we crossed mice that are heterozygous for a Brachyury mutation [39] and assessed expression of the two genes. In wild-type embryos at E7.5 the expression patterns of Brachyury, Wnt3a and Axin2 overlap significantly (Fig. S4). Expression of Wnt3a in Brachyury homozygous mutant embryos at this stage resembles that in heterozygous and wild type individuals, as has been reported previously [40], but by E8.5, when Wnt3a expression is restricted to the primitive streak, its expression is significantly down regulated in Brachyury mutant embryos (Fig. 4C,D).

Like Wnt3a, Axin2 is expressed in Brachyury mutant embryos at 7.5 dpc, but this expression is more variable than that of Wnt3a, and is sometimes reduced or even absent (data not shown). By E8.5, when Axin2 is expressed in the headfold, tailbud and primitive streak of wild type embryos, its expression in the posterior region of Brachyury mutant embryos is very weak or absent (Fig. 5C,D). Together, these data indicate that Brachyury is
Figure 3. Components of the Wnt pathway as Brachyury targets. (A) The Wnt signalling pathway. Arrows indicate positive interactions and bars represent negative interactions. Targets identified in this study are outlined in **bold**. (B–E) Brachyury binding in genomic regions around Dkk1 (B); Ctnnb1/β-catenin (C); Dvl3 (D); and γ-Catenin/jup/plakoglobin (E). Each target shows fold enrichment against chromosomal position. Blue bars represent the T box-like site TSACANNT (N = any base, S = G/C) and green bars represent (AC)n. Stars above bars represent sequence on the reverse strand. Plots are average of triplicate chip results, aligned to the mm8 Feb. 2006 assembly.

Figure 4. Analysis of Wnt3a, a positive regulator of the Wnt pathway. (A) Location analysis of Wnt3a. The figure (and Figs. 5, 6) shows fold enrichment against chromosomal position. Plot is the mean of triplicate chip results, aligned to the mm8 Feb. 2006 assembly. Blue bars represent the T box-like site TSACANNT (N = any base, S = G/C); green bars represent (AC)n; red bars the consensus TCACACCT. Stars above bars represent sequence on reverse strand. (B) Quantitative RT-PCR expression profile for Wnt3a during ES cell differentiation, expressed relative to beta actin. (C, D) Expression of Wnt3a studied by in situ hybridisation; in each, the top image shows a dorsal view, and the bottom image a lateral view. (C) Phenotypically wild type (+/+) or (+/+T) embryo at E8.5–8.75, and (D) a mutant (T/T) embryo from crosses of Brachyury heterozygous mutant mice. Wnt3a expression is detected with NBT/BCIP (purple) and the insets show a lateral view after double staining for Brachyury detected with INT/BCIP (orange brown). Note that in the wild type embryo Wnt3a is expressed in tailbud and paraxial mesoderm. In the mutant embryo expression of Wnt3a staining is absent or greatly reduced (n = 3). Scale bars indicate 250 μm.

doi:10.1371/journal.pone.0033346.g003

doi:10.1371/journal.pone.0033346.g004
required for the proper expression of Wnt3a and Axin2, which encode key components of the WNT signalling pathway (see Discussion).

**Fgf8 as a target of Brachyury**

A strong Brachyury binding peak was also detected 5' of the transcription start site of Fgf8 (Fig. 6A), with a variant Brachyury site (5'-TCAGAGAT-3'; underlined bases differ from consensus) positioned 63 nucleotides from an (AC)19 repeat. The temporal expression profile of Fgf8 resembles that of Brachyury during embryoid body differentiation (Fig. 6B), and the gene is co-expressed with Brachyury in the primitive streak of embryos at E7.5 and E8.0–E8.25 (Fig. S3; Fig. 6C). Expression of Fgf8 in mutant Brachyury embryos at E8.0 is greatly reduced (Fig. 6C), indicating that Brachyury is required for expression of this gene as it is for Wnt3a and Axin2.
Figure 6. Fgf8 as a target of Brachyury. (A) Location analysis of Fgf8. For details of methods see legend to Fig. 4. (B) Quantitative RT-PCR expression profile for Fgf8 during ES cell differentiation, expressed relative to beta actin. (C) Expression of Fgf8 studied by in situ hybridisation. The images show a phenotypically wild type (+/+ or +/+T) embryo (top pair) and a mutant T/T (bottom pair) embryo derived from crosses of Brachyury heterozygous mutant mice. The wild type embryo is orientated with anterior to the left and posterior to the right; the mutant is viewed from the posterior. Fgf8 expression is detected with NBT/BCIP (purple) and Brachyury with INT/BCIP (orange brown). In the wild type embryo Fgf8 is expressed in the primitive streak and paraxial mesoderm; such expression is absent or greatly reduced in the mutant. Scale bars indicate 200 μm.

doi:10.1371/journal.pone.0033346.g006
AXIN2, JUP, FGF8 and WNT3A are conserved targets of BRACHYURY in the human

Because some of the Brachyury targets we discovered in the mouse were not previously identified in the frog or the zebrafish [16], we decided to investigate whether these are conserved in other species, in an attempt to further validate our results. For this purpose we decided to look for BRACHYURY binding in the human genome.

We have recently optimised culture conditions that cause human embryonic stem cells to differentiate into mesoderm-like cells [41]. These cell populations express BRACHYURY at high levels and, importantly, they also up regulate other mesoderm markers, including many of the Brachyury targets we have identified in the mouse, namely, DKK1, HOXA13, ID4, JUP, KRT8, MEIS1, MSGN1, SNAI2, and WNT3A.

We therefore made use of this newly developed in vitro differentiation system to ask if BRACHYURY binds the homologous human regulatory regions of some key mouse targets: AXIN2, FGF8, JUP and WNT3A. As in the mouse, these regions contain imperfect T-binding motifs (data not shown). Our experiments involving ChIP-qPCR with hESC-derived mesoderm cells indeed detected a strong enrichment for these sequences, thus indicating that BRACHYURY binds to the same genomic regions in the human (Fig. 7A).

Interestingly, these promoter sequences seem to be conserved between the mouse and human genomes, but not in the zebrafish or in other vertebrates (Fig. 7B) suggesting that these targets might be unique to mammals.

Discussion

We have identified genomic targets of Brachyury in differentiating mouse ES cells, demonstrating that embryoid bodies provide sufficient material for chromatin immunoprecipitation experiments and that they represent an effective model of early mouse development. Although they do not undergo proper morphogenesis, they do generate pattern, as illustrated by the formation of beating cardiomyocytes [42]. The embryoid bodies produced in our experiments form cardiomyocytes after eight to ten days in culture, close to the time at which the heart tube forms during normal development. As discussed below, at least three Brachyury targets (Wnt3a, Axin2 and Fgf8) are expressed in the early mouse embryo during formation of the primitive streak, and their proper expression during development requires Brachyury function. We also note that several targets are expressed in primordial germ cells, perhaps the first differentiated population to emerge during early gastrulation [43,44,45]. These cells express Brachyury until E12.5, when the gene is down regulated in a non-migrating population [46,47]. Although Brachyury may not be involved directly in the specification of the germ cells [48,49] it may regulate their migration and their potency.

Classification of Brachyury targets and comparison with zebrafish and frog

Our work has identified 396 potential targets of Brachyury, and gene ontology analysis indicates that many of these encode sequence-specific DNA binding proteins and proteins involved in cell adhesion and embryonic morphogenesis. Analysis of the former category will help in the elucidation of the genetic regulatory network that underlies mesoderm formation (see below). The latter category includes cell junction proteins and glycosyltransferases [31], consistent with the finding that the extracellular matrix of homozygous mutant Brachyury mouse embryos is poorly developed [32] and that cells have fewer

![Figure 7. Conservation of BRACHYURY binding in the human genome.](http://example.com)
cytoplasmic processes, both of which may contribute to the failure of mutant cells to move out of the primitive streak and to the failure of elongation of the antero-posterior axis [30]. Amongst the other genes regulated by Brachyury are those encoding cytokines and components of signal transduction pathways, and in most of these respects our results are reminiscent of those obtained in similar experiments using the zebrafish embryo [16].

It is significant that embryoid bodies resemble developing embryos in this way, and it is also important to note the overlap between the Brachyury targets identified in this study and the Ntl targets identified in the zebrafish [16]. Both studies identified transcriptional regulators as being enriched, including members of the homeobox, winged helix, paired box, zinc finger and odd-paired families. There are also similarities in the functions of genes regulated by the two orthologues. These functions include gastrulation (where the zebrafish study identified wnt11, snail1a and blf and this analysis Wnt3a, Snail2 and genes such as Gdf5, Ets1, Krt5, Krt8, Lmx1b, Syk, and Gnaq); muscle specification (where both studies identified Msgn1 and Pax3); posterior identity (where the zebrafish study identified fgfr4, fgfr2b, cent, vox, and notch3 and this analysis Fgfb [50]) and left-right patterning (zebrafish genes include cxv3.4 [51] and our mouse targets Rtn [52], Fgfb [53], and cytoplasmic dyneins Dynch1b, Dynch2b [54] and Dped [55]).

The fact that there are some differences between the mouse and zebrafish targets may derive from the presence of an additional Brachyury gene in the zebrafish genome [8] or from the ‘sharing’ of gene function between different T box family members. For example, the Bst genes were identified as targets of Brachyury and VegT in Xenopus [13,14], but their mouse ortholog Mex1 seems to be regulated mainly by Eomesoderm [56,57]. Furthermore, as illustrated in Fig. 7B, some regulatory sequences of mammalian genes (Axin2, Fgfb, Jup and Wnt3a) share little homology with those of their zebrafish orthologues. It is possible that Brachyury binds different locations in different genomes, which has been noted for other transcription factors [58]; despite target conservation. It is also likely that Brachyury binds not only to promoters near the gene transcription start site but also to distant enhancers [59], which is indeed the case in the human genome (T. Faial et al., in preparation). We note that both the mouse and zebrafish arrays were based on promoter regions, so that enhancer binding is not available in these datasets, perhaps explaining why some targets seem to be unique to each species.

Canonical and non-canonical T-box binding sites

Our previous work searching for targets of zebrafish Ntl showed that the canonical T-box site TCACACCT was enriched in the vicinity of Ntl target genes [16]. A significant enrichment of this motif was not observed in the present experiments for the majority of targets. Rather, we identified a novel (AC)ₙ repeat sequence that recognised, albeit weakly, the Brachyury T domain in electrophoretic mobility shift experiments. We do not yet fully understand the significance of this observation. Mouse Brachyury binds to an imperfect T-box site palindrome in the Nanog promoter [60], but no other Brachyury target has been characterised in any detail in this species. It is possible that mouse Brachyury resembles Drosophila Brachyteron, where modular variations on the T-box consensus binding sequence determine the degree of transcriptional activation [61]. A similar system controls notochord formation in Ciona, with regulatory motifs comprising Ciona-Brachyury and Cg-foxA binding sites [62].

Moreover, many transcription factors bind directly to DNA in distal enhancer elements [59], and are then linked to the promoter region by chromatin looping, allowing interaction with other proteins involved in transcription regulation [59]. It is likely that in some mouse targets, canonical Brachyury binding motifs are not present in the promoter region but rather in upstream or downstream regulatory regions. Our results show that this does occur in the human genome (T. Faial et al., in preparation).

It is also possible that the AC repeats cause the transient formation of left handed DNA helices and bends, changing the chromatin architecture and encouraging transcription factor binding [63]. Brachyury may be an example of a protein with a secondary recognition motif [64] and that the presence of both an AC repeat and a TCAGACCT sequence allows stable binding that cannot be competed by an excess of just the TCAGACCT sequence (Fig. S3B). Repetitive sequences may also function as pre-sites; that is, as regions of DNA that are predisposed to evolve into new regulatory sequences [65].

Brachyury modulation of Wnt and Fgf signalling

Several components of the Wnt signal transduction pathway were identified as Ntl targets in the zebrafish, and we find that the same is true for Brachyury in the mouse. In an effort to determine whether Brachyury regulates expression of these potential targets during normal mouse development we asked whether Wnt3a and Axin2 are expressed normally in Brachyury homozygous mutant embryos, and found that although both genes are expressed at E7.5 (albeit rather variably in the case of Axin2), neither is expressed at E8.5 in mesodermal derivatives (Figs. 4, 5). This suggests that Brachyury is not required for the initial activation of Wnt3a or Axin2, but is needed for maintenance of their expression. Together with the observation that Wnt3a maintains Brachyury expression in the early mouse embryo via TCF/Lef signalling [20,21], and that Axin2 is down regulated in Wnt3a mutants [66], our data indicate that Brachyury and Wnt signalling cooperate to create a regulatory network that specifies the formation of posterior mesoderm in the mouse embryo.

Part of this network may involve Fgf signalling. Brachyury and Fgf signalling form part of an autoregulatory loop in Xenopus and zebrafish embryos [67,68,69,70], and we note that Fgfb is a target of Brachyury in embryoid bodies, and that its expression is down regulated in Brachyury mutant embryos (Fig. 6).

Finally, our work reveals that the promoter regions of Axin2, Fgfb and Wnt3a are also bound by BRACHYURY (Fig. 7A) in human ES cells as they differentiate into mesoderm-like cells [41]. These results further substantiate the identity of these genes as bona fide Brachyury targets and suggest that the regulation of these key signalling components is conserved during human development.

Making a genetic regulatory network for mesoderm

Attempts to understand the Brachyury genetic regulatory network are important not only because Brachyury is required for proper formation of mesoderm in the vertebrate embryo, but because it is sufficient for the formation of some mesodermal cell types, at least in Xenopus [71]. The identification of new Brachyury targets will enable the integration of Brachyury with other components of genetic regulatory networks that include it, such as the Ets family member Elk-1 and the caudal homologue, Cdx2 [72] and to ask to what extent such networks have been conserved during evolution.

Materials and Methods

Ethics statement

Animal procedures were performed under a UK Home Office project license within the conditions of the Animals (Scientific Procedures) Act 1986.
Mouse ESC culture

Embryonic stem (ES) cell culture was as described [73] except that mitotically inactivated primary mouse embryonic fibroblasts (MEFs) were used as feeders. Culture dishes were coated with 0.1% gelatin (Sigma-Aldrich). MEFs and ES cells were maintained in DMEM (Sigma-Aldrich) supplemented with 0.1 mM β-mercaptoethanol, non-essential amino acids (Gibco Invitrogen), 2 mM glutamate (Gibco Invitrogen), and batch-tested 10% (MEFs) or 15% (ES cells) foetal bovine serum (FBS) (Gibco Invitrogen). ES cell medium was also supplemented with Leukaemia Inhibitory Factor (LIF) (ESGRO®; Millipore) at 10⁴ units/ml [74]. Early passage R1 mouse ES cells [75] were passaged every 2 days and medium was changed daily to prevent differentiation.

ES cells were differentiated in spinner flasks to produce large numbers of embryoid bodies (EBs) undergoing synchronized differentiation [76,77]. The Cellspin culture system (Integra Biosciences) was set at 25 rpm and spin angle 720° so as to avoid aggregation of EBs. Spinner medium was prepared as above, but with LIF omitted and FBS increased to 20%. On day 0, adherent log phase ES cell colonies were dissociated and resuspended in 10 ml spinner medium. Feeder cells were depleted by differential sedimentation at 37°C for 20 min.

Medium (45 ml) was pre-equilibrated in 100 ml silicon-coated (Sigmacote, Sigma-Aldrich) spinner flasks (Integra Biosciences). ES cells were recovered from the gelatin-coated differential sedimentation plates and centrifuged at 800 g for 5 min. Cells were fully dissociated to ensure that cultures were initiated from single cells, and each spinner flask was inoculated with 10⁶ cells in 5 ml medium. After 24 h (day 1 of differentiation) a further 50 ml FlCyB medium was added to each flask. Each day thereafter EBs were allowed to sink and 50 ml medium was aspirated and replaced with 50 ml fresh pre-warmed spinner medium.

Human ESC culture

Human ESCs [H9 (WiCell, Madison, WI)] were maintained and differentiated as previously described [41]. Briefly, hESCs were induced to express BRACHYURY by culturing them in a chemically defined medium (CDM) supplemented with FGF2 (20 ng/ml), LY294002 (10 μM) and BMP4 (10 ng/ml) (termed FlCyB medium). Cells were collected for ChIP after 36 h of culture in FlCyB medium, when BRACHYURY expression peaked.

Quantitative RT-PCR

Gene expression was analysed by real-time RT-PCR. RNA was isolated from differentiated EBs using TRI-Reagent LS (Sigma-Aldrich), digested with DNA-free DNAase I (Ambion), and checked for integrity using an Agilent Technologies 2100 Bioanalyzer. cDNA was generated from 1 μg RNA using Superscript III Reverse Transcriptase (Invitrogen, Life Technologies), and this was followed by real-time PCR using the LightCycler 480 SYBR Green I master kit (Roche). Mouse beta actin primers were used as an endogenous control to express relative expression levels (Table 86).

Antibodies

Several anti-Brachyury antibodies were tested for use in this work. Of these, the goat polyclonal C19 antibody (SC-17745, Santa Cruz Biotechnology), raised against a C-terminal sequence of human Brachyury, performed best in chromatin immunoprecipitation. This antibody, raised against a divergent region of Brachyury that does not include the T box, has been well characterised in previous studies [78,79,80]. It gave the expected pattern of staining in early mouse embryos (Fig. S6A,B) and recognised Brachyury protein (of the correct size) in immunoprecipitation experiments followed by western blots (Fig. S6C). Such experiments failed to detect Brachyury in ES cells in which Brachyury expression was inhibited by use of ShRNA constructs (Fig. S6D,E).

Whole-mount in situ hybridization

Wild type mouse embryos were collected from MF1 or 129 strains, and Brachyury mutant embryos from BTBR T+T/J×BTBR T+T/J heterozygote crosses [39]. Embryos were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), after which they were dehydrated and stored in 100% methanol at −20°C. The mouse Brachyury coding sequence was subcloned into pC2S+ and used to generate a probe. An Atto32 probe was generated from IMAGE clone 1361800 (Geneservice), a WaST probe from IMAGE clone pENTR2231 100015899 after subcloning into pC2S+ (Table S7), and an Fgβ probe from IMAGE clone 6513131 (Geneservice) in pCMV-SPORT 6.1. Digoxygenin labelled or fluorescein labelled antisense RNA probes were generated using T7 RNA polymerase from linearised templates and whole mount in situ hybridisation was performed as described [81]. Alkaline phosphatase was detected using (i) BM purple; (ii) 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (250 μg/ml) plus magna phosphatase (250 μg/ml) (INT/Mag); or (iii) nitro blue tetrazolium (175 μg/ml) plus 5-bromo-4-chloro-3-indolyl phosphate (337.5 μg/ml) (NBT/BCIP) (Roche). These gave dark blue, orange brown or purple staining respectively. A final concentration of 5% polyvinyl alcohol (Sigma-Aldrich) was used in the staining reaction.

Whole-mount immunohistochemistry

Embryos were fixed as described above and rehydrated to PBS for staining. Free aldehyde groups were blocked using 1 M glycine, embryos were washed in PBS/0.1% Tween 20 (PBST), and endogenous peroxidases were blocked using 3% hydrogen peroxide in PBS. Embryos were incubated overnight at 4°C in 1:400 C19 antibody in PBST supplemented with 0.2% bovine serum albumin (BSA) and 10% heat inactivated FBS. They were then washed, incubated with 1:400 rabbit anti-gt-biotinylated IgG (E0466, Dako), and stained using Vectastain Elite ABC substrate (Vector laboratories) with Sigma Fast Nickel Enhanced DAB chromagen (Sigma).

In vitro translation and western blotting

Brachyury mRNA was synthesized using the pC2S+ construct described above and the Ambion mMessage mMachine (Applied Biosystems/Ambion). mRNA was translated in a rabbit reticulocyte lysate (Promega). In vitro translation products and embryoid body extracts were subjected to polyacrylamide gel electrophoresis (PAGE) and western blots were performed using CAPS transfer buffer (10 mM CAPS pH 11, 10% Methanol). Membranes were blocked with 5% milk powder in PBST overnight, and antibodies were diluted in the same solution. Washes were in PBST. Primary antibodies were R&D Systems anti-T and SantaCruz anti-T (see above). Both were used at a dilution of 1:250. Secondary antibodies were HRP-linked SantaCruz D anti-goat IgG (1:20,000) and HRP-linked Amersham NA934V anti-rabbit IgG (1:100,000). All antibody incubations were 1 hour at room temperature. Both endogenous and in vitro translated T proteins were immunoprecipitated for western blotting using the Santa Cruz Exactacruz D anti-goat system (SC-45041, Santa Cruz) to avoid detection of heavy and light chains of the IP antibody. Detection used the Pierce Supersignal West Dura Extended Duration Substrate (Thermo Scientific).
Chromatin immunoprecipitation (mouse ESCs)

Chromatin immunoprecipitation/location analysis was based on the Agilent Mammalian ChiP-chip Protocol, incorporating the Whole Genome Amplification GenomePlex Kit (Sigma) [82]. Intact EBs (1.5 × 10⁶ cells) were fixed in 1 M formaldehyde for 20 min when Brachyury expression was at its highest level (usually after 4 days of differentiation). This was followed by quenching and isolation of nuclei. Our protocol differs from a previously-published procedure [83] in that EBs are not disrupted before fixation. Nuclei were sonicated using a Misonix 3000 ultrasonicator to create fragments of 500 bp, and these were immunoprecipitated using polyclonal goat anti-Brachyury C-19 (Santa Cruz Biotechnology) or normal goat IgG (Santa Cruz Biotechnology) as an isotype control. Following washing and elution steps, cross-links were reversed overnight at 65°C. Samples were analysed by promoter-specific primers or amplified by GenomePlex whole genome amplification for microarray studies.

Chromatin immunoprecipitation (human ESCs)

ChIP was performed as previously described [83] with some modifications. Briefly, H9 hESCs (one confluent 10 cm dish) were collected after 36 hr of culture in FLyB medium [41], when BRACHYURY expression peaked. Cells were fixed as described [83], the nuclei were isolated and sonicated using a Misonix 4000 to obtain DNA fragments of around 1000 bp. Samples were incubated at 4°C overnight using 10 μg of an anti-BRACHYURY goat IgG (R&D systems) and with10 μg of a non-specific goat IgG as a control. The chromatin was immunoprecipitated by adding 100 μl of Protein G Dynabeads (Invitrogen), then incubating at 4°C 1 h, and collecting the beads using a magnetic rack. After washing the beads, the chromatin was eluted and the crosslinking was reversed at 65°C overnight. Samples were then treated with RNase and Proteinase K and the DNA was extracted by phenol/ chloroform, ethanol-precipitated and finally eluted in nuclelease-free water. This experiment was repeated three times with similar results.

Verification of target enrichment was performed on a selection of targets using genomic quantitative PCR. DNA fragments were Amplified using Fast SYBR® Green Master Mix (Applied Biosystems) according to manufacturers instructions on a 7500 Fast Real-Time PCR System (Applied Biosystems) to assess the significance of enrichment ratios. Target probes and surrounding promoter sequences were scanned for the published consensus in vitro T-box binding motif TCACACCT [17,18] using NestedMICA http://www.sanger.ac.uk/Software/analysis/nmica/index.shtml [85]. We also used Regulatory Sequence Analysis Tools (RSAT) http://rsat.ulb.ac.be/rsat/ [86], to scan each target gene over a region −5 kb to +1 kb relative to its ATG for over-represented cis-regulatory modules, applying background models and taking promoter sequences from 400 random mouse promoters as the control set. Sequences representing enriched motifs were then stacked into positional weight matrices and converted to sequence logos using WebLogo (http://weblogo.berkeley.edu/logo.cgi) [87].

Bioinformatic analyses and Motif Finding

The GOTOolBox [84] was used to access Gene Ontology (GO) resources and to search for any functional bias in our dataset. The Benjamin and Hochberg multiple testing correction was applied to assess the significance of enrichment ratios. Target probes and surrounding promoter sequences were scanned for the published consensus in vitro T-box binding motif TCACACCT [17,18] using NestedMICA http://www.sanger.ac.uk/Software/analysis/nmica/index.shtml [85]. We also used Regulatory Sequence Analysis Tools (RSAT) http://rsat.ulb.ac.be/rsat/ [86], to scan each target gene over a region −5 kb to +1 kb relative to its ATG for over-represented cis-regulatory modules, applying background models and taking promoter sequences from 400 random mouse promoters as the control set. Sequences representing enriched motifs were then stacked into positional weight matrices and converted to sequence logos using WebLogo (http://weblogo.berkeley.edu/logo.cgi) [87].

Electrophoretic mobility shift assays

The T domain of mouse Brachyury was amplified by PCR (primer sequences in Table S7) and inserted in-frame into the glutathione-S-transferase (GST) fusion vector pGEX-6P-1. The fusion protein was expressed in E. coli by isopropyl-β-D-thiogalactoside induction and purified at 4°C using GSTrap FF columns and Pre-Scission Protease (GE Healthcare), leaving only a glycine and a proline residue attached to the protein. This was concentrated using Amicon Ultra 4 columns (Millipore), and the identity of the resulting protein was confirmed by SDS PAGE and mass spectrometry.

Double stranded oligomers containing (i) the core Brachury consensus binding sequence TCACACCT, (ii) a simple AC repeat, or (iii) the core Brachury sequence together with the AC repeat, and mutated versions of each, had identical BglII/BamH1 5’ overhangs (Table S7). These were PAGE purified, annealed, and end-labelled with [α-³²P]dCTP using Klenow fragment. Unincorporated nucleotides were removed using Sephadex G-50 columns (GE Healthcare). Binding reactions were incubated on ice for 40 minutes in 1 x EMSA binding buffer (23 mM HEPES pH8.0, 100 mM KC1, 1 mM DTT, 0.1% NP-40, 5% glycerol, 10 mM EDTA), 0.5% milk powder and 50 ng/μl d1/dC using 30,000 cpm/μl and 8–10 fmol labelled oligomer. Competition reactions using 4 pmol cold oligomers were pre-incubated for 10 min on ice. In supershift experiments goat polyclonal anti-Brachyury N19 antibody (SC-17743, Santa Cruz) was added after binding and then incubated a further 20 min on ice.
Supporting Information

Figure S1 Validation of targets. Box plot showing genomic quantitative PCR of bound promoter regions for targets *Axin2*, *Fox1*, *Mayer2*, *Nkx2.6*, *Pax3*, *Rtn*, *Van Gogh* and the published target *Nanog*, and unbound or negative promoter regions *Nanog* 3’, 1700010C24Rik and *beta actin*. Boxes represent the interquartile range, the upper edge being the 75th percentile and lower edge the 25th percentile. The whiskers show the minimum and maximum values. Values above the line are enriched in chromatin immunoprecipitations. Data were obtained from five independent chromatin immunoprecipitations. Probes recognising *Nanog* were not present on Agilent 244K promoter arrays.

(TIF)

Figure S2 Functional analysis of target genes. Bar charts show Gene Ontology (GO) annotations for (A) biological process; (B) cellular component; and (C) molecular function using the GOToolBox. Horizontal bars represent enrichment ratio (observed frequency/expected frequency) and vertical axis gives the GO term followed by the GO identification number in brackets (arrow) and tailbud. Staining was absent in controls in which primary or secondary antibodies were omitted. (B) Expression of *Brachyury* mRNA in an E9.5 embryo studied by in situ hybridisation. Note similarity to (A). Bars in (A) and (B) represent 250 μm. (C) Western blot testing antibody specificity. Size markers are shown to the left. Lane 1: Mouse Brachyury reticulocyte lysate translation product; lane 2: unprogrammed reticulocyte lysate translation product; lane 3: Immunoprecipitated material derived from Brachyury reticulocyte lysate translation product; lane 4: Supernatant of immunoprecipitated material in lane 3; lane 5: Immunoprecipitated material derived from day 4 embryoid bodies; lane 6: Supernatant of immunoprecipitated material in lane 5; lane 7: Immunoprecipitated material derived from day 4 embryoid bodies, having omitted first antibody; lane 8: Supernatant of immunoprecipitated material in lane 7. All immunoprecipitations used Santa Cruz anti-T C19. Western blots used R&D Systems anti-T as a primary antibody and SantaCruz D anti-goat IgG HRP linked secondary antibody. (D) Strategy to create ES cell clones lacking Brachyury. Clones were created using 65 bp ShRNA duplexes targeting the first exon of *Brachyury* (T). Sequences were inserted into the Xhol/HindIII site of the pSingle ShRNA vector (Clontech) which includes a tetracyclin-controlled transcriptional repressor that in turn regulates the expression of the ShRNA sequence. Selection of stable lines is achieved by culture in G418 and induction of ShRNA expression occurs through addition of 1 μg/ml doxycycline. (E) Western blot analysis of day 5 embryoid body extracts from clones containing ShRNA constructs targeted to Brachyury exon 1 (T1) or a scrambled version of this sequence (Ts), either treated with doxycycline (+) or left untreated (−). Samples were immunoprecipitated as in (C). Note loss of Brachyury band in lane 3.

(TIF)

Table S1 Full gene list.

(DOC)

Table S2 Targets involved in key signalling pathways.

(DOC)

Table S3 Genes associated with germ cell development.

(DOC)

Table S4 Targets identified as transcription factors.

(DOC)

Table S5 Conservation of AC repeats.

(DOC)
Table S6 Quantiative PCR primers.

(DOC)

Table S7 In situ hybridisation, T box, and electrophoretic mobility shift assay primers.

(DOC)

Table S8 Genomic quantitative PCR primers.

(DOC)

Acknowledgments

We thank Valerie Wilson (Centre for Regenerative Medicine, Edinburgh) for the mouse T plasmid. We are grateful to our colleagues for helpful discussions throughout the course of this work, and especially James Smith and Rick Livesey (Gordon Institute) for their advice concerning Chip-chip experiments.

Author Contributions

Conceived and designed the experiments: ALE TF RAP LV FCW JCS. Performed the experiments: ALE TF. Analyzed the data: ALE TF FCW MJG TD JCS. Contributed reagents/materials/analysis tools: LV RAP. Wrote the paper: ALE TF FCW JCS.

References

1. Herrmann BG, Labeit S, Poustka A, King TR, Lehrach H (1990) Cloning of the T gene required in mesoderm formation in the mouse. Nature 343: 617–622.
2. Wilkinson DG, Bhati S, Herrmann BG (1990) Expression pattern of the mouse T gene and its role in mesoderm formation. Nature 343: 657–659.
3. Dobrovolskaià-Zavadskaià N (1927) Sur la mortification spontanée de la queue chez la souris nouveau-née et sur l’existence d’un caractère (facteur) heréditaire. C R Seances Soc Biol Fil 97: 114–116.
4. Nairhe LA, Harrison Z, Kelly RG, Papaoanou VE (2005) T-box genes in vertebrate development. Annu Rev Genet 39: 219–239.
5. Yanagisawa KO, Fujimoto H, Urushiba H (1981) Effects of the Brachyury (T) mutation on morphogenetic movement in the mouse embryo. Developmental Biology 77: 72–78.
6. Smith JC, Price BM, Green JB, Weigel D, Herrmann BG (1991) Expression of a Xenopus homolog of Brachyury (T) is an immediate-early response to mesoderm induction. Cell 67: 79–87.
7. Schulte-Merker S, Ho KK, Herrmann BG, Nuslein-Volhard C (1992) The progenitor product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. Development 116: 1021–1032.
8. Martin BL, Kimmel D (2008) Regulation of Canonical Wnt Signaling by Brachyury T: Essential for Posterior Mesoderm Formation Development. Cell 13: 121–133.
9. Herrmann BG, Kispert A (1994) The T genes in embryogenesis. Trends in Genetics 10: 280–286.
10. Conlon FL, Sedgwick SG, Weston KM, Smith JC (1996) Inhibition of Xhra transcription activation causes defects in mesodermal patterning and reveals autorregulation of Xhira in dorsal mesoderm. Development 122: 2427–2435.
11. Schulte-Merker S, van Erden FJ, Halpern ME, Kimmel CB, Nusslein-Volhard C (1994) No tail (ntf) is the zebrafish homologue of the mouse T (Brachyury) gene. Development 120: 1009–1015.
12. Casey ES, O'Reilly MA, Conlon FL, Smith JC (1998) The T-box transcription factor Brachyury regulates expression of eGFR through binding to a non-palindromic response element. Development 125: 3807–3894.
13. Casey ES, Tada M, Fairclough L, Wylie CC, Heasman J, et al. (1999) Btc is activated directly by VegT and mediates endoderm formation in Xenopus development. Development 126: 4193–4200.
14. Tada M, Casey ES, Fairclough L, Smith JC (1998) Btc, a direct target of Xbra-T genes, causes formation of ventral mesoderm and endoderm. Development 125: 3997–4006.
15. Tada M, Smith JC (2000) Xwnt11 is a target of Xenopus Brachyury: regulation of gastulation movements via Dsh/cedulin, but not through the canonical Wnt pathway. Development 127: 2227–2236.
16. Merlot RH, Lachani K, Kreft D, Gilchrist MJ, Fleck P, et al. (2009) A gene regulatory network directed by zebrafish No tail accounts for its roles in mesoderm formation. Proceedings of the National Academy of Sciences 106: 3829–3834.
17. Kispert A, Herrmann BG (1993) The Brachyury gene encodes a novel DNA binding protein. Embry J 12: 3211–3220.
18. Kispert A (1995) The Brachyury protein: A T-domain transcription factor. Seminars in Developmental Biology 6: 395–403.
19. Long JY, Kollip FI, Wu R, Zhai Y, Knick R, et al. (2002) Activation of ANX2 expression by beta-catenin-T cell factor. A feedback repressor pathway regulating Wnt signaling. J Biol Chem 277: 21657–21665.
20. Yamaguchi TP, Takada S, Yoshikawa Y, Wn N, McMahon AP (1999) T (Brachyury) is a direct target of Wnt3a during paraxial mesoderm specification. Genesis Dev 15: 315–320.
21. Galceran J, Hsu S, Grosschedl R (2001) Rescue of a Wnt mutation by an activated form of LEF-1: Regulation of maintenance but not initiation of Brachyury expression. Proceedings of the National Academy of Sciences 1015123088: 898.
22. Aulkerla A, Wehrle C, Brand-Saberi B, Kunderer R, Gossler A, et al. (2005) Wnt3a plays a major role in the segmentation clock controlling somitogenesis. Dev Cell 4: 395–406.
23. Valamehr B, Jonas SJ, Polleux J, Qiao R, Guo S, et al. (2008) Hydrophilic surfaces for enhanced differentiation of embryonic stem cell-derived embryoid bodies. Proc Natl Acad Sci U S A 105: 14459–14464.
24. Wing L, Shin EH, Grosschedl R, Page-McCaw A, et al. (2007) Expression of Msg1 in the presomite mesoderm is controlled by synergy of Wnt signalling and Tbx5. EMBO Rep 8: 704–709.
25. Tampini O, Kanzel D, Cox B, Bell C, Rossant J, et al. (2000) Microarray analysis of Foxc2 mutant mouse embryos reveals novel gene expression and inductive roles for the gastrula organizer and its derivatives. BMC Genomics 9: 511.
26. Sousa-Nunes R, Rana AA, Kettleborough R, Brickman JM, Clements M, et al. (2003) Characterizing embryonic gene expression patterns in the mouse using non-redundant sequence-based selection. Genome Res 13: 2690–2696.
27. Elms P, Scarry A, Davies J, Willoughby C, Hacker T (2004) Overlapping and distinct expression domains of Zic2 and Zic3 during mouse gastrulation. Gene Expression Patterns.
28. Weinstein DC, Ruiz-I Altaba A, Chen WS, Hoodless P, Prezioso VR, et al. (1994) The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo. Cell 78: 575–586.
29. Kurisaki T, Masuda A, Oumi N, Nabeeshima Y, Fujiwara-Schwarz A (1998) Spatially- and temporally-regulated gene expression controlling the organizer in zebrafish embryos. Genes Dev 12: 3829–3834.
30. Wilson V, Manson L, Skarnes WC, Beddington RS (1995) The T gene is necessary for normal mesodermal morphogenetic cell movements during gastrulation. Development 121: 877–886.
31. Shur BD (1982) Cell surface glycosyltransferase activities during normal and mutant (T/T) mesenchyme migration. Dev Biol 91: 149–162.
32. Jacobs-Cohen RJ, Speigelman M, Bennett D (1984) Abnormalities of cells and extracellular matrix of T/T embryos. Differentiation 25: 40–55.
33. Popsueva AE, Luchinskaya NN, Sudicky AV, Zinovjeva OY, Poteryaev DA, et al. (2001) Overexpression of camello, a member of a novel protein family, reduces blastomere adhesion and inhibits gastrulation in Xenopus laevis. Dev Biol 234: 463–496.
34. Liu D, Watabiki A, Bayyan J, Zhang F, Liu L, et al. (2000) ASAP1, a Gene at 8q24, Is Associated with Prostate Cancer Metastasis. Cancer Res 60: 4352–4359.
35. Armstrong J, Pritchard-Jones K, Bickmore W, Hastie N, Baird J (1992) The expression of the Wilms' tumour gene, Wt1, in the developing mammalian embryo. Mechanisms of Development 40: 85–97.
36. Garnett AT, Han TM, Gilchrijt MJ, Smith JC, Eisen MB, et al. (2009) Identification of direct T-box target genes in the developing zebrafish mesoderm. Development 136: 749–760.
37. Conlon FL, Fairclough L, Price BMJ, Casey ES, Smith JC (2001) Determinants of T-box protein specificity. Development 128: 3749–3758.
38. Kispert A, Koschorz B, Herrmann BG (1995) The T protein encoded by Brachyury is a tissue-specific transcription factor. Embry J 14: 4763–4772.
39. King T, Beddington RS, Brown NA (1998) The role of the brachyury gene and beta (ADAM12) in mouse embryogenesis. Mech Dev 73: 211–215.
40. Wilson V, Manson L, Skarnes WC, Beddington RS (1995) The T gene is necessary for normal mesodermal morphogenetic cell movements during gastrulation. Development 121: 877–886.
41. Bernardo AS, Faha T, Gardner L, Niakan KK, Ortmann D, et al. (2011) BRACHYURY and CDX2 mediate BMP-induced differentiation of human and mouse pluripotent stem cells into embryonic and extraembryonic lineages. Cell 145: 85–97.
42. Lawon KA, Hage WJ (1994) Clonal Analysis of the Origin of Primordial Germ Cells in the Mouse. In: Joan Marsh JG, ed. Ciba Foundation Symposium 182 - Germline Development. pp 68–91.
46. Clements D, Taylor HC, Herrmann BG, Stott D (1996) Distinct regulatory control of the Brachyury gene in axial and non-axial mesoderm suggests separation of mesoderm lineages early in mouse gastrulation. Mechanisms of Development 56: 139–149.

47. Saitou M, Barton SC, Surani MA (2002) A molecular programme for the specification of germ cell fate in mice. Nature 418: 293–300.

48. Lawson KA, Dunn NR, Roelen BA, Zeinstra LM, Davis AM, et al. (1999) BMP1 is required for the generation of primordial germ cells in the mouse embryo. Genes Dev 13: 424–436.

49. Ohinata Y, Ohha H, Shigeta M, Yamanaka K, Wakayama T, et al. (2009) A signaling principle for the specification of the germ cell lineage in mice. Cell 137: 571–584.

50. Crossley PH, Martin GR (1996) The mouse Egfl gene encodes a family of poly peptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. Development 121: 439–451.

51. Esner JJ, Laing JG, Beyer EC, Johnson RG, Hackett PB, Jr. (1996) Expression of zebrafish connexin3 in the notochord and tail bud of wild-type and mutant no tail embryos. Dev Biol 177: 448–462.

52. Faisst AM, Alvarez-Bolado G, Treichel D, Gruss P (2002) Rotatin is a novel gene required for axial rotation and left-right specification in mouse embryos. Mechanisms of Development 113: 15.

53. Tsang TK, Knodt SJ, Tran PF (1999) Experimental analysis of the emergence of left-right asymmetry of the body axis in early postimplantation mouse embryos. Cell Mol Biol (Noisy-le-grand) 45: 493–503.

54. Rana AA, Barbera JP, Rodriguez TA, Lynch D, Hirst E, et al. (2004) Targeted deletion of the novel cytoplasmic dynamin mD2/LIC disrupts the embryonic organiser, formation of the body axes and specification of ventral cell fates. Development 131: 4999–5007.

55. Zarivala M, O’Neal WK, Noonie PG, Leigh MW, Knowles MR, et al. (2004) Investigation of the Possible Role of a Novel Gene, DPCD, in Primary Ciliary Dyskinesia. Am J Respir Cell Mol Biol 30: 428–434.

56. Pearce JJ, Evans MJ (1999) Mml, a mouse Mlx-like gene expressed in the primitive streak. Mech Dev 87: 189–192.

57. Russ AP, Wältler S, Colledge WH, Aparicio SAJR, Carlton MBL, et al. (2000) Eomesoderm is required for mouse trophoblast development and mesoderm formation. Nature 404: 95–99.

58. Schmidt D, Wilson MD, Ballester B, Schwalie PC, Brown GD, et al. (2010) Five-vertebrate ChIP-seq reveals the evolutionary dynamics of transcription factor genomic targets. Nature reviews Genetics 10: 605–616.

59. Ohinata Y, Ohta H, Shigeta M, Yamanaka K, Wakayama T, et al. (2009) A signaling principle for the specification of the germ cell lineage in mice. Cell 137: 571–584.

60. Suzuki A, Raya A, Kawakami Y, Morita M, Matsui T, et al. (2006) Nanog binds to Smad1 and blocks bone morphogenetic protein-induced differentiation of embryonic stem cells. Proc Natl Acad Sci U S A 103: 10294–10299.

61. Kusch T, Storck T, Walldorf U, Reuter R (2002) Brachyury proteins regulate formation. Nature 404: 95–99.

62. Passamaneck YJ, Katikala L, Perrone L, Dunn MP, Oda-Ishii I, et al. (2008) Direct activation of a notochord cis-regulatory module by Brachyury and FoxA to promote posterior mesodermal development. Development 135: 4639–4654.

63. Isaacs HV, Fowshall ME, Slack JM (1994) eFGF regulates Xbra expression during Xenopus gastrulation. EMBO J 13: 4469–4481.

64. Schulte-Merker S, Smith JC (1995) Mesoderm formation in response to Brachyury requires FGF signalling. Cell Biol 5: 62–67.

65. Draper BW, Stock DW, Kimmel CB (2005) Zebrafish fgf24 functions with fgf8 to promote posterior mesodermal development. Development 130: 4639–4654.

66. Griffin KJ, Kimelman D (2005) Interplay between FGF, one-eyed pinhead, and T-box transcription factors during zebrafish posterior development. Dev Biol 284: 436–446.

67. Cunlliffe V, Smith J (1992) Ectopic mesoderm formation in Xenopus embryos caused by widespread expression of a Brachyury homologue. Nature 358: 427–430.

68. Neutwich O, Dingwall KS, Nordheim A, Smith JC (2009) Downstream of FGF during mesoderm formation in Xenopus: the roles of Eek-1 and Efg-1. Dev Biol 336: 313–326.

69. Evans A, Bryant J, Skeeper J, Smith S, Print CG, et al. (2007) Vascular development in embryoid bodies: quantification of transgenic intervention and antiangiogenic treatment. Angiogenesis 10: 217–226.

70. Williams RL, Hilton DJ, Pease S, Willson TA, Stewart CL, et al. (1988) Myeloid leukemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 336: 684–687.

71. Nagy A, Rossant J, Nagy R, Abramov-Newerly W, Rodier JC (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci U S A 90: 8424–8428.

72. Wartenberg M, Gunther J, Hescheler J, Sauer H (1998) The embryoid body as a novel in vitro assay system for antiangiogenic agents. Lab Invest 78: 1301–1314.

73. Thomson M, Liu SJ, Zou LN, Smith Z, Meisner A, et al. (2011) Pluripotency factors in mouse embryonic stem cells regulate differentiation into germ layers. Cell 145: 875–889.

74. Van Eynde A, Nuyten M, Deswerchin M, Schoonsjens L, Peppens S, et al. (2004) The nuclear scaffold protein NIPPP1 is essential for early embryonic development and cell proliferation. Molecular and cellular biology 24: 5803–5814.

75. Bernemann C, Greber B, Ko K, Sterneckert J, Han DW, et al. (2011) Distinct developmental ground states of embryonic stem cell lines determine different pluripotency features. Stem Cells 29: 1496–1503.

76. Wilkinson DG (1992) Whole mount in situ hybridization of vertebrate embryos. In: Wilkinson DG, ed. In situ hybridization: A Practical Approach. Oxford: IRL Press. pp 75–83.

77. O’Green H, Nicolet CM, Balañak K, Green R, Farnham PJ (2006) Comparison of sample preparation methods for ChIP-chip assays. Biotechniques 41: 577–580.

78. Brown S, Teo A, Paulkin S, Naman C, Cho CH, et al. (2011) Actinin/Nodal signaling controls divergent transcriptional networks in human embryonic stem cells and in endoderm progenitors. Stem Cells 29: 1176–1183.

79. Martin D, Bres C, Remy E, Mestres P, Thieffry D, et al. (2004) GFOToolbox: functional analysis of gene datasets based on Gene Ontology. Genome Biol 5: R101.

80. Nentwich O, Dingwell KS, Nordheim A, Smith JC (2009) Downstream of FGF during mesoderm formation in Xenopus: the roles of Eek-1 and Efg-1. Dev Biol 336: 313–326.

81. Bernemann C, Greber B, Ko K, Sterneckert J, Han DW, et al. (2011) Distinct developmental ground states of embryonic stem cell lines determine different pluripotency features. Stem Cells 29: 1496–1503.

82. Wilkinson DG (1992) Whole mount in situ hybridization of vertebrate embryos. In: Wilkinson DG, ed. In situ hybridization: A Practical Approach. Oxford: IRL Press. pp 75–83.

83. O’Green H, Nicolet CM, Balañak K, Green R, Farnham PJ (2006) Comparison of sample preparation methods for ChIP-chip assays. Biotechniques 41: 577–580.

84. Martin D, Bres C, Remy E, Mestres P, Thieffry D, et al. (2004) GFOToolbox: functional analysis of gene datasets based on Gene Ontology. Genome Biol 5: R101.

85. Down TA, Hubbard TJP (2005) NestedMICA: sensitive inference of over-represented motifs in nucleic acid sequence. Nucleic Acids Res 33: 1445–1453.

86. Crooks GE, Hon G, Chandonia J-M, Brenner SE (2004) WebLogos: A Sequence Logo Generator. Genome Res 14: 1188–1190.