The Role of Mga in the Survival of Pluripotent Cells During Peri-implantation Development

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ABSTRACT

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The dual specificity transcription factor $Mga$ contains both a T-box binding domain and a basic helix-loop-helix zipper (bHLHZip) domain. Loss of $Mga$ leads to embryonic lethality by E5.5. In vitro blastocyst culture and embryonic stem (ES) cell culture identify a lack of pluripotent inner cell mass (ICM) derived cells as the cause of embryonic lethality. Loss of $Mga$ leads to increased apoptosis in E4.5 embryos, though there is no decrease in the amount of cell proliferation. Embryos with mutant $Mga$ have fewer pluripotent ICM cells during delayed implantation, though the number of differentiated primitive endoderm cells remained initially stable. Despite the loss of pluripotent cells, there is no change in the pattern of expression of Nanog or Oct4, pluripotent cell markers, or Gata4, a primitive endoderm marker. Expression of Ornithine Decarboxylase (ODC), the rate-limiting enzyme in the synthesis of cellular polyamines, was identified as a possible cause of embryonic lethality based on a similar mutant phenotype as well as the presence of E-box sequences in genetic regulation loci. ODC is expressed at lower levels in the ICM of $Mga$ mutants. Blastocyst and ES cell culture defects were rescued when cultured in the presence of exogenous putrescine, the metabolic product of ODC. These results suggest a mechanism for Mga to influence
pluripotent cell survival through interactions with other bHLHZip domain proteins in the regulation of the polyamine pool in pluripotent cells of the embryo.
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**Abbreviations**

α-difluoromethylornithine (DFMO)
Basic Helix-Loop-Helix Zipper domain (bHLH-Zip)
Definitive Endoderm (DE)
Embryonic Day (E)
Embryonic Stem (ES)
Extraembryonic Endoderm (ExEn)
Inner cell mass (ICM)
Ornithine Decarboxylase (ODC)
Primitive endoderm (PE)
T-Box Binding Element (TBE)
Trophectoderm (TE)
Zygotic Genome Activation (ZGA)
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Development of the preimplantation embryo

Preimplantation development of the mouse embryo occurs over the first 4.5 days of embryonic life. During this time, the embryo goes through radical changes on both a morphological and gene expression level to establish the three major cell lineages that will contribute to further development after implantation. When the embryo implants between 4.5 and 5.5 days post coitus (defined as E for embryonic day), there are three major cell lineages that will contribute to the embryo: the epiblast (Epi), the trophectoderm (TE), and the primitive endoderm (PE). The Epi gives rise to all of the tissues that make up the body of the embryo. It remains undifferentiated and pluripotent largely through the action of Pou5f1 (referred to here by its common name, Oct4), Nanog, and Sox2 (Avilion et al., 2003; Loh et al., 2006; Nichols et al., 1998; Silva et al., 2009). Pluripotency relies both on the repression of differentiation factors as well as the activation of pluripotency factors. In contrast, the PE and the TE have already undergone initial stages of differentiation and will eventually give rise to most of the extraembryonic structures present during gestation. Development and differentiation of embryonic tissues relies on tight transcriptional control of the embryonic genome.

Activation of the zygotic genome

Transcription in the fertilized egg initially relies on maternally-derived mRNA transcripts deposited during oogenesis. It is not until the late one-cell/2-cell stage that an initial wave of zygotic transcription occurs (Hamatani et al., 2004). This wave of transcription, collectively referred to as the zygotic genome activation (ZGA), is both short-lived and is accompanied by rapid downregulation of ZGA-target genes and degradation of target transcripts. The zygotic genes that become active during initial ZGA are characterized as coding for basic cellular machinery including ribosomal proteins, RNA binding, proton transport, among others. Simultaneously, during the time of ZGA, maternal transcripts are degraded rapidly, allowing the zygotic genome to
become the main source of genetic instructions for further development (Paynton et al., 1988).

It is not until a second wave of transcription from the 4-cell stage to the 8-cell stage that genes specific for development of the pre-implantation embryo begin to be transcribed. While transcription of these genes is transient before being immediately decreased, these are the genes that have been shown to be responsible for patterning the pre-implantation embryo. This wave of transcription includes genes such as *Pou5f1* and *Nanog*, pluripotency factors in the ICM, as well as genes such as *Gata3* and *Irx3*, markers of trophoblast (Tanaka et al., 2002).

The presence of maternal transcripts is essential for development, but is often a complicating factor in the analysis of early gene function. Many genes are essential for embryonic development, but are deposited in the egg as either transcript or protein before ZGA. This makes loss-of-function studies difficult as the embryonic genome may contain loss-of-function alleles while the maternally derived transcripts or proteins are functional. This will mask potential embryonic phenotypes while the maternal transcript or protein persists.

**Differentiation of the trophectoderm**

The earliest zygotic cell divisions and the activation of the zygotic genome leave all daughter cells with the same developmental totipotency. The first cell lineage restriction is the differentiation of the trophoblast, the tissue that will become the placenta. Initiation of differentiation begins when cells of the 8-cell zygote divide asymmetrically to generate “inner” and “outer” populations of cells (Johnson and McConnell, 2004). During this time, “outer” cells that will eventually become TE upregulate the homeobox gene *Cdx2* (Jedrusik et al., 2008). *Cdx2* appears to be the key factor for the differentiation of trophoblast as embryos mutant for *Cdx2* do not downregulate the ICM markers *Oct4* and *Nanog* in the presumptive trophoblast cells resulting in the death of these “outer” cells (Strumpf et al., 2005). *Cdx2* also plays a role in maintaining the structural integrity of the embryo via E-cadherin adherins junctions present in the trophoblast during blastocyst formation (Strumpf et al., 2005). *Eomesodermin (Eomes)* also plays a role in proliferation of the trophoblast, though
mutants lacking *Eomes* are still able to form a TE layer suggesting that it acts downstream of *Cdx2* (Teo et al., 2011).

Conversely, *Oct4* and *Nanog* are initially expressed throughout the embryo, but eventually become restricted to the ICM by the action of trophoblast and TE differentiation genes such as *Cdx2* and *Eomes*. Both *Oct4* and *Nanog* have been shown to be repressors of early differentiation: *Oct4* represses trophoblast differentiation and *Nanog* represses extra-embryonic endoderm and PE (Mitsui et al., 2003; Nichols et al., 1998). In this way, there is a feedback mechanisms where cells of the trophoblast downregulate ICM genes, and cells of the ICM downregulate trophoblast genes.

*Differentiation of the primitive endoderm*

The second cell lineage restriction in the embryo is the formation of the PE, a monolayer of cells underlying the Epi that will eventually give rise to the yolk sac. The differentiation of the PE begins at E3.5 when cells of the ICM begin to differentially express the pluripotency marker *Nanog* and the PE marker, *Gata6* in a “salt and pepper” pattern (Chazaud et al., 2006). *Gata6* is upregulated by Fgf signaling through the adaptor protein *Grb2*, though the mechanism for targeting only a subset of ICM cells is not understood (Chazaud et al., 2006). Tracking of presumptive PE cells using another marker of PE, *Pdgfrα*, shows that they can either migrate to the PE monolayer or undergo apoptosis, suggesting a role for cell adhesion molecules in the sorting and survival of PE cells (Plusa et al., 2008). Once PE cells are formed, they rely on the Epi to survive as Nanog-deficient embryos are able to initially form a PE, which then degenerates (Silva et al., 2009).

*The T-box transcription factors in preimplantation embryonic development and pluripotency*

The necessity of transcriptional control of differentiation during preimplantation development suggests that different families of transcription factors can contribute to this process. The T-box transcription factors are an ancient and evolutionarily conserved family of transcription factors necessary for a large number of developmental processes
(Naiche et al., 2005). In mouse, there are 17 T-box factors that can be subdivided into 5 subfamilies based on sequence similarity. All of the T-box factors share a common DNA binding domain, the T-box, which can bind the T-box binding element (TBE), a palindromic sequence with two T-half sites (5’-AGGTGTGAAATT-3’) (Kispert and Herrmann, 1993). Dimers of Brachyury are able to bind this sequence, with each monomer binding one T-half site of the TBE (Papapetrou et al., 1997). Different T-box factors bind their respective T-sites in various homo- and heterodimeric combinations, as well in competition with each other or other transcription factors (Habets et al., 2002; Sinha et al., 2000).

Although detected in other organisms as early as the oocyte stage of development, most T-box factors are only functionally necessary during gastrulation and organogenesis (Gibson-Brown et al., 1998; Greulich et al., 2011; Showell et al., 2004). Nonetheless, there is evidence of a role for two T-box genes during blastocyst formation and in the maintenance or acquisition of pluripotency.

**Tbx3**

*Tbx3* transcripts are first detected in the ICM of E3.5 blastocysts (Bollag et al., 1994; Chapman et al., 1996). Despite this expression, the role of *Tbx3* in the ICM is unclear. Homozygous loss-of-function *Tbx3* mutants have no phenotype at E3.5, though there is 50% embryonic lethality by E11.5 with the rest dying by E16.5, most likely due to yolk sac or cardiac deficiency (Davenport et al., 2003; Mesbah et al., 2008).

Nonetheless, *Tbx3* is critical for the function of embryonic stem (ES) cells. Similar to their *in vivo* analog, the ICM, undifferentiated ES cells express *Tbx3*. This expression decreases as the ES cells differentiate implicating *Tbx3* in the maintenance of pluripotency (Lu et al., 2011). This function is supported by the detection of *Tbx3* in a pluripotency transcriptional module involving factors such as Oct4 and Nanog that is able to activate transcription of pluripotent factors and repress differentiation genes (Kim et al., 2008). Moreover, ectopic *Tbx3* expression from a viral plasmid leads to increased efficiency of derivation of induced pluripotent stem cells (iPSC) (Han et al., 2010). Rather than promoting pluripotency, though, *Tbx3* appears to function similarly to *Oct4*.
and Sox2 by repressing differentiation, in this case differentiation into mesoderm, ectoderm, and neural crest cell fates (Ivanova et al., 2006).

In contrast to its role in repression, Tbx3 is also able to promote differentiation of ES cells into extraembryonic endoderm (ExEn). Overexpression of Tbx3 in ES cells induces differentiation of ExEn cells as gauged both by morphology and Gata6 expression (Lu et al., 2011). This duality suggests that, like other factors involved in pluripotency, Tbx3 renders ES cells poised to differentiate when the proper signals are received. These transcription factors act as repressive factors when ES cells are to remain pluripotent, but can quickly switch to activators when differentiation is induced (Bernstein et al., 2006; Washkowitz et al., 2012).

**Eomesodermin**

The only other T-box transcription factor that has been shown to play a role in preimplantation development is Eomes. Eomes is first detected in the trophoblast lineage at E3.5 and continues to be expressed in its derivative, the extraembryonic ectoderm of postimplantation embryos (Hancock et al., 1999; Russ et al., 2000). Eomes homozygous mutations are embryonic lethal soon after implantation due to a defect in trophoblast development and failure of formation of trophoblast stem cells. Eomes likely acts downstream of the homeobox factor Cdx2 in the proliferation and development of the trophoblast (Strumpf et al., 2005).

In the pluripotent ICM and Epi, Eomes is repressed by Nanog, Sox2, and Oct4. Later, during gastrulation, Eomes promotes the differentiation of the embryonic germ layers by repressing mesoderm and pluripotency genes and activating definitive endoderm (DE) genes (Teo et al., 2011). Eomes also regulates the E-cadherin-mediated epithelial-to-mesenchymal transition critical for cell movement and mesoderm specification, thus providing cues for cell specification along the anterioposterior axis (Arnold et al., 2008).
The Max network of basic-helix-loop-helix-leucine zipper transcription factors in early embryonic development and pluripotency

Another group of transcription factors known to play a role in preimplantation development is the basic-helix-loop-helix-leucine zipper (bHLHZip) domain genes of the Max network. The Max network proteins are transcription factors that bind the canonical DNA sequence, the E-box (5’-CACGTG-3’) (Grandori et al., 2000; Hurlin and Huang, 2006). This network is composed of the bHLHZip genes Max and Mga, as well as members of the Myc, Mad, and Mnt families of genes. Most of these genes were identified by the binding of their gene products to Max, highlighting the centrality of Max in the function of this network (Ayer et al., 1993; Hurlin et al., 1999; Hurlin et al., 1995; Meroni et al., 1997; Zervos et al., 1993).

Max lies at the center of this transcriptional network and is required for all proteins of the network to function. Though Max alone is able to homodimerize and bind the canonical E-box, it is transcriptionally inert. Similarly, the other members of this family are unable to bind DNA alone leaving them also transcriptionally inert as monomers. It is only through heterodimerization with Max that the other proteins in the network are able to activate or repress transcription of their E-box-containing target genes. In this way, each protein’s activity is modulated by the presence or absence of the other proteins in the network (Baudino and Cleveland, 2001; Meroni et al., 2000; Walker et al., 2005). Mga is unique among the members of the bHLHZip domain family members because of the presence not just of the bHLHZip DNA binding domain, but a T-box binding domain as well (Hurlin et al., 1999).

The importance of this network was first noted in 1981 when the amplification and overexpression of the proto-oncogene c-Myc was found in Avian Leukosis Virus (ALV)-induced lymphoid leucosis (Hayward et al., 1981). Myc has since been linked to more than 40% of human cancers (Zeller et al., 2003). Despite their importance in a variety of cellular contexts, only a subset of the Max-interacting transcriptional network appears to play a role in early embryonic development.
Max

In both mouse and zebrafish, Max transcripts are found ubiquitously throughout development (Domashenko et al., 1997; Schreiber-Agus et al., 1993). In mouse, there are maternal stores of Max in unfertilized eggs, as well as zygotic Max produced in all cells of the embryo throughout development (Shen-Li et al., 2000).

Despite its near universal expression, zygotic Max appears to be dispensable for development through the preimplantation period, though the maternal stores of the protein could abrogate a need for newly produced Max. Homozygous Max mutant embryos are recovered at a mendelian frequency at E3.5, but are recovered at a lower rate at E6.5 and are not recovered at all by E8.5. Mutant embryos at E6.5 are 50-70% smaller than controls and have no demarcation between embryonic cell layers and no morphologically distinct embryonic features. Mutant embryos also have lower proliferation rates at E6.5, and though there is no accompanying apoptosis, it is possible that earlier waves of apoptosis were missed during analysis (Shen-Li et al., 2000).

The importance of Max during the peri-implantation period suggests a necessity for active Max network transcription factors, though functional redundancy and maternal protein complicate the analysis.

c-Myc

c-Myc is first transcribed at the 4-cell stage and continues to be expressed until the formation of the blastocyst (Domashenko et al., 1997). The importance of this early expression is unclear as c-Myc homozygous mutant mice survive until E10.5 when they die with abnormalities in the heart, pericardium, neural tube, and other structures (Davis et al., 1993). When c-Myc deletion is confined to the Epi, embryos die before E12 of severe anemia and functionally defective hematopoietic stem/progenitor cells, but have none of the structural deficiencies in the formation of the heart or neural tube (Dubois et al., 2008). The difference in embryonic lethality when c-Myc is deleted in the Epi-derived tissues alone compared to the Epi- and TE-derived tissues suggests separate developmental roles for c-Myc: The lack of c-Myc in the TE-derived placenta could lead to placental insufficiency and contribute to the E10.5 lethality of mutant c-Myc embryos,
while the lack of a hematopoietic stem-cell population in the embryo could account for the later lethality of the Epi-restricted mutant c-Myc embryos.

Complementary to c-Myc’s role in the development of a multipotent hematopoietic stem-cell population, c-Myc has also been found to expressed in pluripotent ES cells (Murphy et al., 2005), though its role there is unclear. c-Myc mutant ES cells are grossly normal and c-Myc is not part of the core pluripotency network of Oct4, Sox2, and Nanog (Davis et al., 1993; Kim et al., 2008). Nonetheless, c-Myc’s importance in pluripotency is evident as efficient derivation of iPS cells with c-Myc is not possible (Takahashi and Yamanaka, 2006). This discrepancy may be explained by the presence of the closely related protein N-myc in ES cells. Indeed, ES cells that have both mutant c-Myc and mutant N-myc do not maintain pluripotency or self-renewal, highlighting a necessary role for these genes in ES cells (Varlakanova et al., 2010).

c-Myc has also been proposed to form the core of a transcriptional module that serves to amplify global transcription in ES cells (Nie et al., 2012). In activated lymphocytes, c-Myc was shown to bind to the promoters of virtually all genes that were upregulated compared to non-activated lymphocytes, regardless of whether they contained E-boxes in them. The binding of c-Myc to promoters of expressed genes with or without E-boxes correlated with an increase in their expression levels in ES cells. This binding was also associated with an increase in RNA polymerase II. Because c-Myc-mediated amplification of transcription was not limited to genes with E-boxes in their promoters, c-Myc may function as a global transcriptional modulator for the entire genome (Nie et al., 2012). This functionality is also present in T-lymphocytes and Burkitt’s lymphoma cells suggesting a universal mechanism for c-Myc–mediated gene regulation (Lin et al., 2012).

N-myc

As with c-Myc, the closely related transcription factor N-myc is also thought to play a role in preimplantation development. N-myc transcripts were observed at low levels throughout the E6.5 embryo to the exclusion of the TE (Downs et al., 1989). Later, N-myc transcripts were detected during gastrulation and embryonic time points thereafter, notably in the developing nervous system. (Stanton et al., 1992). Though not
examined earlier in the embryo, *N-myc* is also expressed in ES cells, suggesting that expression may be found in the ICM (Sawai et al., 1991).

The role of *N-myc* in pluripotency and the development of the early embryo is unclear. Multiple studies using different mutant alleles have found that embryos with homozygous *N-myc* mutations are embryonic lethal during organogenesis (Sawai et al., 1993; Stanton et al., 1992), suggesting that *N-Myc* is dispensable during earlier stages. However, as with *c-Myc*, ES cells that carry disrupted versions of both *c-Myc* and *N-myc* do not maintain pluripotency or self-renewal (Varlakhanova et al., 2010). Derivation of iPS cells is also normal when *N-myc* is substituted for *c-Myc* (Blelloch et al., 2007). Moreover, the transgenic replacement of the coding sequence of *c-Myc* with that of *N-myc* results in viable and fertile mice with no apparent defects, highlighting a redundant role for these two genes (Malynn et al., 2000).

*Mga*

*Mga* (MGI: 1352483, synonyms C130042M01Rik, D030062C11Rik, Mad5), the least studied of the Max-network of transcription factors, is also thought to play a role in the development of the embryo. *Mga* was first identified in a yeast two-hybrid screen for interacting partners of Max. *Mga* was identified using E9.5 and E10.5 cDNA libraries and the full length sequence was constructed using an E14.5 kidney cDNA library (Hurlin et al., 1999). *Mga* has since been found to span 73kb of genomic DNA on chromosome 2 and contain 24 exons. The mature RNA is ~9kb and codes for a protein of 3006 amino acid residues. There are two splice forms that differ by the inclusion/exclusion of the 14th exon.

Domain analysis reveals the presence not just of a bHLHZip DNA binding domain, but also a T-box DNA binding domain, making *Mga* a dual-specificity transcription factor. Unlike other T-box transcription factors, the T-box in *Mga* is fully encoded within a single exon leading to the hypothesis that it is the product of the retrotransposition of the T-box of a *Tbx6*-related cDNA, the most closely related T-box transcription factor (Lardelli, 2003)(Fig 1).
Figure 1. Phylogenetic analysis of vertebrate T-box sequences. A. ClustalW alignment of translated T-box sequences. Mga and Tbx6 are highlighted in red. B. Phylogenetic tree derived by parsimony analysis of alignment of (A). Mga is highlighted in red. Adapted from (Lardelli, 2003). Hs, Homo sapiens; Mm, Mus musculus; Dr, Danio rerio; Gg, Gallus gallus.
*In vitro*, *Mga* has been shown to bind the TBE as well as the E-box. Binding of *Mga* to the E-box is dependent on Max dimerization, while binding to the TBE can occur either independently of Max or as a dimer with Max. When bound to E-box sites as a heterodimer, Mga is able to act as a transcriptional activator. However, when bound to a TBE, Mga is able to repress transcription when bound alone, but to activate transcription when bound with Max (Hurlin et al., 1999). This duality suggests that Max can regulate the activity of Mga not only by regulating its binding to the E-box, but also by acting as a switch of transcriptional activity on T-box targets.

*Mga* appears to be widely expressed during development. In zebrafish, *Mga* mRNA was detected as a maternal transcript in the fertilized egg at the 1-cell stage and is expressed widely throughout later development (Rikin and Evans, 2010). In mouse, the expression is more restricted with mRNA first detected at E3.5 and later at E6.5 through E10.5 (Hu et al., 2009). *In situ* hybridization localized E3.5 expression to the pluripotent ICM (Yoshikawa et al., 2006), and expression of both RNA and protein has been detected in ES cells, the *in vitro* analog of the ICM (Hu et al., 2009; van den Berg et al., 2010). *Mga* RNA was also detected during organogenesis in a variety of organs including the limb buds, branchial arches, and tail region, though control assays were not shown (Hurlin et al., 1999) and independent verification has not been possible (our unpublished results).

Very few studies have addressed the role of *Mga* during embryonic development. In zebrafish, morpholino depletion of *Mga* in fertilized eggs results in defects in the brain, heart, and gut derivatives, though no common mechanism was found. In the heart, there was an absence of looping that was partially a result of overexpression of *Gata4* transcripts, indicating a transcriptional repression role of *Mga*. The brain, however, was shown to have an increase of apoptosis that was p53 dependent, indicating a cell survival role for *Mga* (Rikin & Evans, 2010).

In mouse, *Mga* appears to have a role in the pluripotency program. In ES cells, Mga has been shown to be in a complex with the pluripotency factor Oct4, a necessary transcriptional activator for pluripotency (Hammachi et al., 2012). When *Mga* is depleted with siRNA, ES colonies showed a decrease in the transcription of *Oct4, Sox2,*
and Nanog suggesting a transcriptional activation role for Mga in ES cells (Hu et al., 2009; van den Berg et al., 2010).

In addition to its role as a transcription factor, Mga may also modulate transcription by recruiting other complexes to target genes. Mga has been found in polycomb repression complexes with PRC1B and E2F-6 in both ES cells and HeLa cells (Illingworth et al., 2012; Ogawa et al., 2002). In HeLa cells, the presence of Mga in this complex allows chromatin modification and silencing of T-box and E-box targets, suggesting that Mga is able to regulate transcription both on a direct transcriptional level as well as an epigenetic level.

**Aims of this research**

Our initial experiments indicated that Mga was critical for peri-implantation development. It is possible that Mga is necessary for peri-implantation development because it regulates key transcriptional targets through its interaction with Max network proteins. We studies how Mga affects preimplantation development using 4 primary systems. First, we used blastocyst culture of embryos lacking Mga to directly observe the growth and development of the peri-implantation embryo in the absence of Mga. Second, we derived ES cells with a conditional Mga allele to observe any effects that mutation of Mga has on the growth and differentiation of ICM analogues. Third, we used immunofluorescence of E4.5 day embryos to assess the expression of cell layer differentiation and characterize the growth and apoptosis of embryos lacking Mga. Finally, we used hormonally induced diapause to assay maintenance of pluripotency in embryos lacking Mga in the absence of the rapid cell division characteristic of peri-implantation development.
Chapter 2
Results

The role of Mga in preimplantation development

Function of the Mga mutant allele

A conditional Mga mutation was generated by the German Gene Trap Consortium (GGTC), Mouse Genome Informatics (MGI) allele Mga<sup>Gr(E153E01)Wrst</sup>. This is a multipurpose allele from which additional alleles can be derived. In the following, the alleles are referred to as Mga<sup>GT</sup> (for gene trap), Mga<sup>Inv</sup> (for FLP-recombinase inverted gene trap) or Mga<sup>Re-inv</sup> (for Cre-recombinase re-inverted gene trap).

The Mga allele was made with a gene trap construct that contains a splice acceptor and β-galactosidase-neomycin resistance (β-geo) fusion protein cassette that is flanked by 4 sets of heterotypic LoxP and FRT sites (Fig 2). In its original Mga<sup>GT</sup> orientation, the upstream exon donates a splice site that is accepted by the gene trap cassette rather than the endogenous allele. This creates a truncated fusion protein that carries a β-geo reporter under the control of the Mga promoter. When treated with Flp recombinase, the cassette is inverted to the Mga<sup>Inv</sup> configuration and the splice acceptor is put in the wrong orientation to accept the upstream splice, allowing the wild type transcript to be produced. When treated with Cre recombinase, the cassette is flipped to the Mga<sup>Re-inv</sup> configuration, once more producing the β-geo fusion protein. These mechanisms allow the Mga<sup>GT</sup> and Mga<sup>Re-inv</sup> alleles to act as mutant reporters and the Mga<sup>Inv</sup> allele to act as a conditional-mutation allele (Fig 2) (Schnutgen et al., 2005).

ES cell clones were isolated from E14Tg2a ES cells (Sv129P2) after retroviral infection using rsFlpRosaβgeo (FlpRBG; www.genetrap.de). The insertion of FlpRBG in intron 3 of Mga was identified by splinkerette PCR (Horn et al., 2007).

Breeding with Mga mutants

All embryos and adults produced during the course of experiments were routinely genotyped by PCR. Mga<sup>GT/+</sup> mice genotyped at weaning were recovered at the expected Mendelian frequency (46/88 from Mga<sup>GT/+</sup> x Mga<sup>+/+</sup> matings; $\chi^2 = 0.18$, p=0.67) and were viable and fertile indicating that the mutant allele did not have a heterozygous or
Figure 2. The gene trap cassette and Mga mutations produced from the FlpRBG cassette. The FlpRBG targeting vector utilizes a gene trap strategy for the creation of a mutant reporter of Mga. The Mga\textsuperscript{GT} allele orients a splice acceptor-\(\beta\)-galactosidase-neomycin resistance cassette (top) to accept the upstream exon’s splice site and create a mutant truncated reporter protein. After treatment with Flp recombinase (which results in inversion, step 1 and excision, step 2), the splice acceptor is no longer in the proper orientation to accept the upstream splice and a wild type transcript is produced. After further treatment with Cre recombinase, inversion (step 3) and excision (step 4) occur to produce the Mga\textsuperscript{Re-inv} allele, which functions like the Mga\textsuperscript{GT} allele: a truncated reporter protein is produced. Adapted from (Schnutgen et al., 2005).
dominant negative effect (Table 1). No $Mga^{GT/GT}$ mice were recovered at weaning in 19 litters from inter se matings of $Mga^{GT/+}$ mice ($0/84; X^2 = 28.64, p<0.0001$) indicating homozygous lethality before that time (Table 1). $Mga^{GT/+}$ mice were also bred with a constitutively active FlpE recombinase-expressing mouse to generate the inverted conditional allele, $Mga^{Inv}$ (Fig 2). $Mga^{Inv/+}$ mice were born at the expected frequency ($7/13$ from $Mga^{Inv/+} \times Mga^{GT/+}$ matings; $X^2 = 0.33, p = 0.56$) indicating that the conditional $Mga^{Inv/+}$ allele did not have a heterozygous or a dominant negative effect (Table 1). Homozygous $Mga^{Inv/Inv}$ mice, however, were recovered at only $\sim50\%$ of the expected frequency ($19/150$ from $Mga^{Inv/+} \times Mga^{Inv/+}$ matings; $X^2 = 13.45, p = 0.001$) indicating that the conditional $Mga^{Inv}$ allele is not fully functional (Table 1). This conditional allele was insufficient to compensate for the $Mga^{GT}$ mutation of a second allele as no $Mga^{GT/Inv}$ mice were recovered ($0/22$ from $Mga^{Inv/Inv} \times Mga^{GT/+}$ matings; $X^2 = 22, p<0.0001$) (Table 1).

The role of Mga during implantation

Dissection of the uteri of females from inter se $Mga^{GT/+}$ matings at E9.5 – E11.5 revealed empty implantation chambers with cellular debris or a few trophoblast giant cells in approximately $\frac{1}{4}$ of the deciduae examined ($6/25$; from the Mendelian ratios, $X^2 = 6.37, p=0.04$). Trophoblast giant cells from one of these decidua were recovered and proved to be $Mga^{GT/GT}$ when genotyped by PCR. Implantation sites with evidence of embryonic lethality were present at E5.5 as well, where approximately $\frac{1}{4}$ of the deciduae dissected were empty ($9/44$; from the Mendelian ratios, $X^2 = 11.69, p=0.003$). Histological examination of whole uteri at E5.5 and E6.5 showed instances of cellular debris with isolated trophoblast giant cells in approximately $\frac{1}{4}$ of the decidual swellings examined ($10/39$ at E5.5; from the Mendelian ratios, $X^2 = 0.55, p=0.46$; $4/10$ at E6.5; from the Mendelian ratios, $X^2 = 1.20, p = 0.27$) (Fig 3). At E4.5, $Mga^{GT/GT}$ embryos were recovered from uterine flushes at approximately $80\%$ of the expected Mendelian frequency ($49/245; X^2 = 10.91, p = 0.004$) and appeared morphologically normal when compared to their wild type or heterozygous littersmates (Fig 4). Similarly, $Mga^{GT/GT}$ embryos were recovered at the expected Medelian frequency at E3.5 ($21/89; X^2 = 1.30, p = 0.51$) and appeared morphologically normal (Table 2). Taken together, these results
Table 1. Genotype distribution at weaning of progeny from Mga mutant crosses. X² and p values were calculated assuming a Mendelian distribution.

<table>
<thead>
<tr>
<th>Mating</th>
<th>Genotype</th>
<th>X², p</th>
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<tr>
<td></td>
<td>+/+</td>
<td>+/-</td>
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<tr>
<td>Mga&lt;sup&gt;+/+&lt;/sup&gt; x Mga&lt;sup&gt;GT/+&lt;/sup&gt;</td>
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<td>42</td>
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<td>93</td>
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<tr>
<td>Mga&lt;sup&gt;GT/+&lt;/sup&gt; x Mga&lt;sup&gt;Inv/Inv&lt;/sup&gt;</td>
<td>N/A</td>
<td>22 Inv/+</td>
</tr>
</tbody>
</table>
Table 2. Genotype distribution of embryos from $Mga^{GT/+} \times Mga^{GT/+}$ crosses. ND indicates that no genotype was obtained though an implantation site (E5.5) or an empty decidua (E9.5-E11.5) was observed. X$^2$ and p values were calculated assuming a Mendelian distribution of recovered embryos.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Genotype</th>
<th>X$^2$, p</th>
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<tr>
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<td>E9.5-E11.5</td>
<td>6</td>
<td>13</td>
</tr>
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</table>
Figure 3. Histological sections of implantation sites at E5.5 and E6.5. Embryo degeneration (B, D) was detected in 10/39 decidual swellings at E5.5 and 4/10 swellings at E6.5 with trophoblast giant cells seen at E5.5 (yellow arrows). Other implantation sites contained morphologically normal embryos (A, C). MB. Maternal blood; Dc. Decidua; Epi. Epiblast; EPC, Ectoplacental cone; PE, Primitive endoderm; ExEc, Extraembryonic ectoderm; EEc, Embryonic ectoderm; Ch. Chorion. Scale bars: 100µm.
Figure 4. Preimplantation embryos at E3.5 and E4.5. ICM, Inner Cell Mass; Epi, Epiblast; Tro, Trophectoderm; ZP, Zona Pellucida; GC, Giant cells.
indicate that $Mga^{GT/GT}$ embryos implant in the uterus but die shortly thereafter; thus it is likely that $Mga$ plays an essential role in the peri-implantation development of the embryo.

**The function of $Mga$ in the development of the ICM**

*Mga expression in the pluripotent tissues of the embryo*

To assess the contribution of $Mga$ to peri-implantation development, embryonic expression of $Mga$ was assessed using the $\beta$-galactosidase reporter functionality of the $Mga^{GT}$ allele. There was robust $\beta$-galactosidase activity in the Epi of $Mga^{GT/+}$ embryos at E5.5, 6.5, and 7.5 to the exclusion of the PE or extraembryonic tissues (Fig 5E-J). Standard X-gal staining was not sufficient for detection in preimplantation stages, but the more sensitive S-gal method for $\beta$-galactosidase detection showed staining in the Epi at E4.5 in $Mga^{GT/+}$ embryos, but not in $Mga^{+/+}$ embryos (Fig 5B, C). While neither X-gal nor S-gal showed staining at E3.5, RT-PCR for $Mga$ in wild type embryos demonstrated expression at E3.5 (Fig 5A, D). This was the earliest time point that $Mga$ was expressed, as RT-PCR detected no $Mga$ expression in embryos at the 1-cell stage (E0.5) or at E2.5 (Fig 5D).

**The role of $Mga$ in ICM development**

To test the capacity of $Mga^{GT/GT}$ for survival and differentiation outside the uterine environment, embryos were isolated at E3.5 from $Mga^{GT/+} \times Mga^{GT/+}$ matings prior to time of death and cultured *in vitro* before genotyping retrospectively by PCR. $Mga^{GT/+}$ cultures were stained with X-gal to assess $\beta$-galactosidase reporter activity. The ICM of the blastocyst cultures showed strong X-gal staining to the exclusion of the trophoblast 48 hours after starting culture, indicating that the *in vitro* culture system initially recapitulated the embryonic expression of $Mga$ in the Epi before vanishing (Fig 6).

While $Mga^{GT/GT}$ embryos (n=11) were able to attach to the tissue culture substrate and form an outgrowth consisting of trophoblast giant cells, the ICM derivatives failed to
Figure 5. Mga expression in embryos using the β-galactosidase reporter and RT-PCR. 
A-C. No β-galactosidase activity was observed in blastocysts at E3.5 using X-gal stain (A). S-gal staining was present in the Epi of E4.5 Mga<sup>+/+</sup> embryos (B), but not in Mga<sup>−/−</sup> embryos (C). D. RT-PCR on pooled embryos did not detect any Mga transcripts at E0.5 or E2.5, but expression was present at E3.5 and at E4.5. E-J. β-galactosidase staining was observed in the Epi of whole mounts at E5.5 (E), E6.5 (G), and E7.5 (I). Paraffin sections (nuclear fast red counterstain) confirm Epi staining of β-galactosidase from E5.5 (F), E6.5 (H), and E7.5 (J) embryos. ExEc, Extraembryonic Ectoderm; Epi, Epiblast; ExEn, Extraembryonic Endoderm. Scale bars: 40µm.
Figure 6. β-galactosidase activity during *in vitro* culture of E3.5 blastocysts. Blastocyst cultures from *Mga*<sup>GT/+</sup> embryos were stained with X-gal. The ICM of the cultures showed β-galactosidase activity after 48 hours of culture reflecting reporter expression. ICM, Inner cell mass; Tro, Trophoblast.
thrive, resulting in smaller ICM outgrowth surface area by 4 days of culture. In contrast to the normal-appearing trophoblast cells, the ICM cells were sparse and did not form the multicellular or cystic structures of their $Mga^{+/+}$ and $Mga^{GT/+}$ littermates (n=35) when examined 4 days after culture (Fig 7A). Quantification of the surface area of the ICM outgrowth showed similar wild type and mutant ICM outgrowths after 2 days of culture before the mutants either stagnated in their growth or contracted by 4 days of culture (Fig 7B, C). In conjunction with the expression data, this indicates a failure of ICM survival as a contributing factor in the embryonic lethality of $Mga^{GT/GT}$ embryos.

The role of Mga in embryonic stem cell culture

To confirm the importance of Mga for the survival of the ICM, we used the in vitro analog of the ICM, ES cells. Because $Mga^{GT/GT}$ ICMs did not grow when cultured, we derived ES cells from embryos carrying the conditional allele $Mga^{Inv/Inv}$ that also harbored the inducible Cre recombinase gene CreERT2 integrated into the Rosa26a locus (de Luca et al., 2005). $Mga^{Inv/Inv}$, CreERT2 ES cells were morphologically indistinguishable from $Mga^{Inv/+}$ ES cells and grew at similar rates when cultured under normal conditions (Fig 8A, B, D). Additionally, $Mga^{Inv/Inv}$, CreERT2 ES cells expressed pluripotency markers Oct4 and Nanog as expected (Fig 8C).

Upon inversion of the conditional allele by addition of 4-hydroxytamoxifen to the culture, however, $Mga^{Inv/Inv}$, CreERT2 colonies were sparser and smaller than either $Mga^{Inv/+}$ cells or $Mga^{Inv/Inv}$, CreERT2 cells treated with ethanol vehicle alone when viewed at low magnification as a population, though the colony morphology was normal in the individual surviving colonies (Fig 9A). Quantification of cell number at 24-hour intervals revealed an approximate 35% decrease in the number of cells in cultures where inversion had been induced when compared to uninduced $Mga^{Inv/Inv}$, CreERT2 and control cell lines (Fig 9B). Genotyping of the surviving colonies showed that there was incomplete inversion of the $Mga^{Inv}$ allele regardless of the length of tamoxifen treatment (Fig 9C). To ensure that the optimal dose was used to induce inversion, colonies were grown in increasing concentrations of tamoxifen. Both $Mga^{Inv/Inv}$, CreERT2 and $Mga^{Inv/+}$ cultures had fewer colonies present as tamoxifen dose was increased past previous experimental levels of 0.5µM, but the amount of inversion in $Mga^{Inv/Inv}$, CreERT2
Figure 7. In vitro culture of E3.5 blastocysts.  

A. ICM outgrowth from *Mga*<sup>GT/GT</sup> embryos appeared smaller than controls at 3 or 4 days of culture by phase contrast microscopy.  

B, C. Measurement of the surface area at 4 days showed that the ICM surface area of *Mga*<sup>GT/GT</sup> embryos was smaller that controls (B), although the trophoblast outgrowth was similar (C).
Figure 8. Growth and differentiation of $Mga^{Inv/Inv}$; CreERT2 ES cells. A-B. ES cell colonies appeared morphologically similar to $Mga^{Inv/+}$ ES cells. C. Immunohistochemistry for normally expressed pluripotency markers Nanog (green) and Oct4 (red) in $Mga^{Inv/Inv}$; CreERT2 ES cells. Nuclei are stained with Hoeschst (blue). D. $Mga^{Inv/Inv}$; CreERT2 ES cells grew at a similar rate to $Mga^{Inv/+}$ ES cells.
Figure 9. ES cells treated with tamoxifen to induce inversion to the mutated allele. **A.** *MgaInv/Inv; CreERT2* ES cells formed smaller and more sparse colonies when treated with 4-hydroxytamoxifen than when untreated, though colony morphology appeared normal in tamoxifen treated cells (A, inset). **B.** *MgaInv/Inv; CreERT2* ES cells grew at a slower rate from the time of tamoxifen addition at t=0. **C.** Surviving colonies showed partial inversion from the *MgaInv* allele to the *MgaRe-inv* allele.
cultures remained constant, indicating that greater levels of inversion could not be induced before tamoxifen levels became toxic to cells (data not shown). Together, these results indicate that Mga is necessary for the survival of ES cells and that the surviving colonies were those that had escaped inversion and thus had at least one functional Mga allele.

**Apoptosis and cell proliferation in Mga<sup>GT/GT</sup> embryos**

Reduced ICM outgrowth of Mga<sup>GT/GT</sup> blastocyst cultures and reduced growth of inversion-induced Mga<sup>Inv/Inv; CreERT2</sup> ES cells suggests an increase in cell death and/or a decrease in cell proliferation in the ICM or ICM derivatives such as the Epi. To investigate cell proliferation, immunofluorescence with antibodies against phosphorylated histone H3 (phospho-H3) was performed. Counts of the number of positive cells in the Epi did not reveal any difference in the number of mitotic cells in Mga<sup>GT/GT</sup> embryos (3.1 +/- 0.8) compared to Mga<sup>+/+</sup> and Mga<sup>GT/+</sup> embryos (2.8 +/- 0.3) at E4.5 (Fig 10B) (t=0.259, p=0.65) (Figure 10C). The appearance of phospho-H3 nuclear staining can also be used to assess the stage of mitosis that cells are in: prophase nuclei have uniform phospho-H3 staining throughout the nucleus while metaphase and anaphase nuclei have more punctate staining indicative of condensed chromatin. The number of cells that were in prophase in Mga<sup>GT/GT</sup> embryos (1.6 +/- 0.5) was not different from the number of cells in Mga<sup>+/+</sup> and Mga<sup>GT/+</sup> embryos (1.8 +/- 0.3). Similarly, the number of cells in metaphase or anaphase in Mga<sup>GT/GT</sup> embryos (1.5 +/- 0.6) was not different from Mga<sup>+/+</sup> and Mga<sup>GT/+</sup> embryos (1.0 +/- 0.2). Together, these data indicate that there is no defect in progression through the cell cycle in Mga<sup>GT/GT</sup> embryos (Fig 11) (Brenner et al., 2003).

To investigate apoptosis, immunofluorescence of E4.5 day embryos with antibodies against cleaved caspase 9, a marker of fragmented nuclei during apoptosis, was performed (Zhu et al., 2012). A greater proportion of Mga<sup>GT/GT</sup> embryos (7/9) had cleaved caspase 9-positive fragmented nuclei than did Mga<sup>+/+</sup> and Mga<sup>GT/+</sup> littermates (7/47) (p=0.012, Fisher’s Exact Probability test) (Fig 10A). There was no difference in the proportion of Mga<sup>+/+</sup> embryos with cleaved caspase 9-positive fragmented nuclei (3/20) compared to Mga<sup>GT/+</sup> embryos (4/27) (p=1.00 Fisher’s Exact Probability test) supporting the lack of a heterozygous embryonic phenotype. The higher
Figure 10. Apoptosis and cell proliferation at E4.5. Numbers indicate the proportion of embryos showing the illustrated expression pattern. A. Immunofluorescence for cleaved caspase 9 shows that more $Mga^{GT/GT}$ embryos had fragmented nuclei than did $Mga^{+/+}$ and $Mga^{GT/+}$ embryos. B. Cell proliferation as measured by phosphorylated histone H3 immunostaining was similar in $Mga^{GT/GT}$ compared to $Mga^{+/+}$ and $Mga^{GT/+}$ embryos. C. Box plots of the number of phospho-histone H3-positive cells showed no difference between $Mga^{GT/GT}$ embryos and $Mga^{+/+}$ and $Mga^{GT/+}$ embryos.
Figure 11. Cell cycle staging in the Epi of embryos at E4.5. A. $Mga^{GT/+}$ embryo stained for phospho-histone H3. Cell cycle stage can be determined by the pattern of phospho-histone H3 staining. Prophase nuclei show uniform staining (yellow arrowheads) compared to metaphase and anaphase nuclei, in which condensed chromatin staining is more punctate (red arrowheads). B. Box plots of the number of cells in meta/anaphase or prophase of mitosis. There was no difference in the number of cells in prophase or metaphase/anaphase in $Mga^{GT/GT}$ embryos compared to $Mga^{+/+}$ and $Mga^{GT/+}$ embryos.
incidence of apoptosis in $Mga^{GT/GT}$ embryos indicates a defect in the normal function of the cell leading to cell death rather than a defect in cell proliferation.

**Differentiation of primitive endoderm in $Mga^{GT/GT}$ embryos**

*Structural integrity of $Mga^{GT/GT}$ embryos*

To test whether the structural integrity of $Mga^{GT/GT}$ embryos was intact, immunofluorescence with antibodies against E-cadherin was performed. E-cadherin forms a thin layer around the compacted Epi and PrE with a thicker layer of staining in the basement membrane of the surrounding TE. $Mga^{GT/GT}$ embryos had a similar staining pattern at E3.5 (n=4) and E4.5 (n=2) (Fig 12A, B) indicating that lethality was not the result of the embryo losing its structure.

*Gene expression and pluripotency during the differentiation of the embryonic cell layers*

At E4.5, just prior to the time that $Mga^{GT/GT}$ embryos die, differentiation of the embryonic cell layers has begun. The pluripotent ICM differentiates into two cell layers: the Epi remains pluripotent and becomes a compacted cell layer while the differentiated PE forms an epithelial sheet beneath it. Immunofluorescence with antibodies against the pluripotency marker Nanog, which marks the Epi, and the PE marker Gata4 did not reveal any difference in the formation of cell layers between $Mga^{GT/GT}$ (n=3) and $Mga^{+/+}$ or $Mga^{GT/+}$ embryos (n=4) at E4.5 (Fig 12C, D). Immunofluorescence with antibodies against Oct4, a marker of pluripotency expressed in the Epi, did not generate sufficient signal to be analyzed, so an Oct4 GFP reporter allele, $Pou5f1^{tm2Jae}$ (Lengner et al., 2007), was bred into the $Mga^{GT}$ background. Immunofluorescence with anti-GFP antibodies showed a compacted Epi in both the $Mga^{+/+}$ and $Mga^{GT/+}$ embryos (n=27) and $Mga^{GT/GT}$ embryos (n=7) carrying the $Pou5f1^{tm2Jae}$ allele (Fig 12E, F). Together, these results indicate that $Mga^{GT/GT}$ embryos showed disruption of the spatial and temporal gene expression normally characteristic of the differentiation of embryonic cell layers. Additionally, these results indicate that $Mga^{GT/GT}$ embryos do not lose expression of pluripotency markers.
Figure 12. Primitive endoderm differentiation in E4.5 embryos. Numbers refer to the proportion of embryos showing the illustrated expression pattern. A, B. Optical sections of E4.5 embryos show that the E-Cadherin (red) staining pattern is similar in Mga$^{GT/GT}$ embryos and Mga$^{+/+}$ and Mga$^{GT/+}$ embryos. C-F. Projections of z-stacks show that Nanog (green C, D) and Oct4 as measured by GFP reporter (green E, F), markers of the pluripotent Epi, are similar in Mga$^{GT/GT}$ embryos compared to Mga$^{+/+}$ and Mga$^{GT/+}$ embryos, as was the primitive endoderm marker Gata4 (red, C-F).
**Maintenance of pluripotent cells in Mga\(^{GT/GT}\) embryos**

Despite the presence of pluripotency markers at E4.5, the death of the pluripotent cells of the Epi and its *in vitro* derivatives suggest that loss of pluripotent cell self-maintenance could be responsible for the lethality of Mga\(^{GT/GT}\) embryos. To test maintenance of pluripotency in Mga\(^{GT/GT}\) embryos, we examined embryos in which diapause was induced (Nichols et al., 2001). Diapause is a natural mechanism of delayed implantation of embryos in mothers who are still nursing a litter, and can be induced experimentally by hormone treatment. During diapause, blastocysts do not implant in the uterus and Epi cells maintain pluripotency but do not undergo the cell proliferation and differentiation evident during normal development.

To determine whether *Mga* is expressed during diapause in normal embryos, RT-PCR of pooled wild-type embryos was done. One day after induction of diapause by tamoxifen and depo-provera injections at E2.5, *Mga* expression is at a level similar to E3.5 embryos before falling and being maintained a low level of expression at 4 days and 7 days of diapause (Fig 13D).

Immunofluorescence using antibodies against the pluripotency marker Nanog, present in the Epi, and the PE marker Gata4 was used to assess the persistence of cell populations throughout diapause. In diapause embryos, similar to the situation in E4.5 day embryos, the Epi forms a compacted group of cells overlying the PE. One day after induction of diapause, Mga\(^{GT/GT}\) embryos showed spatial gene expression patterns of Nanog and Gata4 that were identical to Mga\(^{+/+}\) and Mga\(^{GT/+}\) embryos. The number of Nanog-expressing cells was similar in Mga\(^{GT/GT}\) embryos (16.5 +/- 1.5) compared to Mga\(^{+/+}\) and Mga\(^{GT/+}\) embryos (13.7 +/- 1.2) (z = 1.17, p = 0.24 Mann-Whitney U test) (Table 3). However, by 4 days after diapause induction, the number of Nanog-expressing cells in Mga\(^{GT/GT}\) embryos (5.4 +/- 1.0) was smaller than Mga\(^{+/+}\) and Mga\(^{GT/+}\) embryos (18.1 +/- 0.9) (z = 3.240, p = 0.001) (Table 3). The relative position and number of Gata4-expressing cells, however, was similar in Mga\(^{GT/GT}\) (21.9 +/- 2.8) compared to Mga\(^{+/+}\) and Mga\(^{GT/+}\) embryos (26.6 +/- 1.3)(z = 0.98, p = 0.33) (Fig 13A-C, Table 3). By 7 days after diapause induction, there were virtually no Nanog positive cells in Mga\(^{GT/GT}\) embryos (0.3 +/- 0.3 cells), while in Mga\(^{+/+}\) and Mga\(^{GT/+}\) embryo Nanog-positive cells persisted (12.5 +/- 1.4 cells) (z = 2.39, p = 0.02) (Fig 13D, Table 3). In
Figure 13. Loss of pluripotent cells during hormonally induced diapause. A. Nanog-positive cells (green) are present initially, but are gradually lost during diapause in $Mga^{GT/GT}$ embryos. Gata4-positive cells (red) persist longer but eventually disappear by E2.5+7 days. B, C. The number of Nanog-positive cells is significantly different in $Mga^{GT/GT}$ at 4 and 7 days of diapause (B), while Gata4-positive cells are significantly different only at 7 days of diapause (C). “WT” refers to $Mga^{+/+}$ and $Mga^{GT/+}$ embryos. D. One day after diapause is induced, RT-PCR indicates that in wild type embryos $Mga$ is expressed at levels comparable to E3.5 embryos and then declines during diapause.
Table 3. Numbers of cells in differentiated cell layers in hormonally induced diapause embryos. Nanog is a marker of pluripotent Epi and Gata4 is a marker of primitive endoderm. Average number of cells and standard error were calculated on the indicated number of embryos.

<table>
<thead>
<tr>
<th>Time</th>
<th>Genotype</th>
<th>Nanog-positive cells</th>
<th>Gata4-positive cells</th>
<th>n (embryos)</th>
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<td>E2.5 + 1 day</td>
<td>$Mga^{+/+}$ and $Mga^{GT/+}$</td>
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<td>$Mga^{GT/GT}$</td>
<td>16.5 +/- 1.5</td>
<td>0 +/- 0</td>
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<tr>
<td>E2.5 + 4 days</td>
<td>$Mga^{+/+}$ and $Mga^{GT/+}$</td>
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<td>26.6 +/- 1.3</td>
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<tr>
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<td>$Mga^{GT/GT}$</td>
<td>5.4 +/- 1.0</td>
<td>21.9 +/- 2.8</td>
<td>7</td>
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<tr>
<td>E2.5 + 7 days</td>
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<td>12.6 +/- 1.4</td>
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<td>$Mga^{GT/GT}$</td>
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<td>6.7 +/- 0.9</td>
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contrast to earlier time points, the number of Gata4-positive cells was also reduced in $Mga^{GT/GT}$ embryos (6.7+/-.9) compared to $Mga^{+/+}$ and $Mga^{GT/+}$ embryos (35.7+/-.24) ($z=2.39$, p-.02) (Fig 13A-C, Table 3). The Gata4-expressing cells appeared to have collapsed against the TE in the $Mga^{GT/GT}$ embryos. Moreover, the mutant embryos had cells in the Epi that stained for neither Nanog nor Gata4 (Fig 13D). While formation of embryonic cell layers occurs normally, the declining number of Epi cells in $Mga^{GT/GT}$ embryos during diapause implies that the pluripotent cells of the Epi are not properly maintained without $Mga$. Conversely, the lack of an early effect on Gata4 expressing cells reflects a lack of requirement for $Mga$ in PE differentiation.

**Regulation of downstream targets of $Mga$**

*Expression of Myc-target gene Odc1 in $Mga^{GT/GT}$ embryos*

After confirming proper structural integrity, cell layer differentiation, and pluripotency in $Mga^{GT/GT}$ embryos at E4.5, we utilized a candidate gene approach to assess downstream genetic effects of the $Mga$ mutation (Table 4). Odc1 (MGI: 97402, synonym Odc), the gene coding for ornithine decarboxylase (ODC), shows a similar expression pattern and embryonic mutant phenotype with $Mga$ (Pendeville et al., 2001). ODC catalyzes the decarboxylation of ornithine to form putrescine, the rate-limiting step in the polyamine synthesis pathway for the production of spermine and spermidine (Fig 14A). In addition to the common embryonic phenotype it shares with $Mga$, Odc1 has 2 E-box sites that have been shown to be able to bind bHLH-Zip domain family members including c-Myc and Mnt (Bello-Fernandez et al., 1993; Nilsson et al., 2004) (Fig 14B).

Immunofluorescence was performed on E4.5 embryos with antibodies against ODC. Projections of confocal stacks showed decreased ODC signal in the Epi of $Mga^{GT/GT}$ embryos (4/6) at E4.5 compared to $Mga^{+/+}$ and $Mga^{GT/+}$ embryos (3/31) (p=.006; Fisher’s Exact Probability test) (Fig 14C). There was also strong signal on the exterior of the TE in all samples, though secondary antibody controls (data not shown) indicated that this was background staining.
Table 4. Candidate genes for *Mga* mutant analysis. Candidate genes were chosen based on similar embryonic phenotype of homozygous mutants. Of these candidates, the presence of E-box sites in a regulatory intron of *Odc1* was unique.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutant Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Max</em></td>
<td>Empty deciduae recovered after implantation, ICM failure in blastocyst culture</td>
<td>(Shen-Li et al., 2000)</td>
</tr>
<tr>
<td><em>Klf5</em></td>
<td>Embryos not recovered after implantation, ectopic PE development</td>
<td>(Lin et al., 2010)</td>
</tr>
<tr>
<td><em>Notchless</em></td>
<td>Empty deciduae recovered after implantation, ICM failure in blastocyst culture</td>
<td>(Cormier et al., 2006)</td>
</tr>
<tr>
<td><em>CTCF</em></td>
<td>Empty deicudea recovered after implantation, ICM failure in blastocyst culture</td>
<td>(Moore et al., 2012)</td>
</tr>
<tr>
<td><em>Oct4</em></td>
<td>Empty deicudea recovered after implantation, ICM failure in blastocyst culture</td>
<td>(Nichols et al., 1998)</td>
</tr>
<tr>
<td><em>Odc1</em></td>
<td>Empty deciduae recovered after implantation, ICM failure in blastocyst culture, E-box target sites in regulatory intron</td>
<td>(Pendeville et al., 2001)</td>
</tr>
</tbody>
</table>
Figure 14. Polyamine synthesis pathway and the expression of Ornithine Decarboxylase (ODC) in E4.5 embryos. A. ODC is the rate limiting step in the polyamine synthesis pathway that produces spermine and spermidine. ODC catalyzes the conversion of ornithine to putrescine. Putrescine is then converted into spermidine and spermine with the addition of decarboxylated S-adeonsyl-methionine (dcSAM). B. Two E-box binding sites (CACGTG) are evolutionarily conserved in the second intron. Projections of confocal Z-stacks show lower levels of ODC (red) in the Epi of Mga<sup>GT/GT</sup> embryos at E4.5. Secondary antibody background is present on the TE of all embryos tested.
ICM growth in the presence of exogenous putrescine

Decreased Odc1 expression in Mga\textsuperscript{GT/GT} embryos suggests that embryos may lack the ability to produce putrescine and thus may lack the necessary end products of the polyamine synthesis pathway. Putrescine deficiency caused by inhibition of ODC by the small molecule α-difluoromethylornithine (DFMO) can be alleviated by addition of exogenous putrescine to IE6 intestinal crypt cell cultures (Iwama et al., 1990). Similarly, we attempted to rescue embryonic lethality in Mga\textsuperscript{GT/GT} embryos by supplying exogenous putrescine to embryos. Specifically, we supplemented blastocyst cultures with 200µM putrescine and measured the ICM outgrowths following 96 hours of culture, a time after which Mga\textsuperscript{GT/GT} ICM outgrowths had been shown to be deficient in untreated cultures (Fig 8).

The surface area of the ICM outgrowth of Mga\textsuperscript{GT/GT} blastocyst cultures was significantly larger when embryos were treated with putrescine (n=7) than when they were untreated (n=11) (t = -4.58, p = 0.0003) (Fig 15A, B). The surface area of the ICM outgrowth in Mga\textsuperscript{+/+} and Mga\textsuperscript{GT/+} cultures, however, was similar with (n=29) or without putrescine (n=24) (t=1.4, p=0.18). None of the treatments affected the outgrowth of trophoblast cells in culture. Notably, the ICM surface area of the putrescine-treated Mga\textsuperscript{+/+} or Mga\textsuperscript{GT/+} cultures (n=29) and Mga\textsuperscript{GT/GT} cultures (n=11) was not significantly different (t = -1.83, p=0.08). Morphologically, the ICM of treated mutants appeared to lack the 3-dimentional structural complexity that was present in Mga\textsuperscript{+/+} or Mga\textsuperscript{GT/+} cultures, though they still appeared larger than untreated Mga\textsuperscript{GT/GT} cultures (Fig 15A). Together, these results indicate that exogenous putrescine is sufficient to at least partially rescue the ICM outgrowth of Mga\textsuperscript{GT/GT} embryos.

ES cell growth in the presence of exogenous putrescine

The rescue of the ICM in blastocyst cultures with exogenous putrescine suggests that the ES cell survival defect of inversion-induced Mga\textsuperscript{Inv/Inv; CreERT2} ES cells might also be rescued. ES cells were plated in unsupplemented ES media and allowed to attach for 1 day. Cultures were then given ES media that was either unsupplemented, contained tamoxifen to induce inversion, or contained both tamoxifen and 200µM putrescine. Cultures were allowed to grow for an additional 2 days before cells were counted.
Figure 15. Blastocyst and ES cell culture with or without 200µM putrescine added. A, B. ICM outgrowths of Mga$^{GT/GT}$ blastocyst cultures appeared larger when treated with putrescine for 4 days (A). ICM surface area after 4 days of culture of treated Mga$^{GT/GT}$ cultures was greater than untreated Mga$^{GT/GT}$ cultures and was not different from treated Mga$^{+/+}$ or Mga$^{GT/+}$ cultures (B). C. Greater numbers of Mga$^{inv/inv}$; CreERT2 ES cells that had inversion induced were present after 2 days of culture when treated with putrescine than when untreated.
Cell counts from $Mga^{inv/inv}; CreERT2$ cultures treated with tamoxifen were higher when treated with putrescine than when untreated ($t = 6.14; p < 0.0001$), but not as high as untreated $Mga^{inv/inv}; CreERT2$ ES cells ($t = 3.17; p = 0.0036$), suggesting that other factors may be required to compensate for the loss of $Mga$ (Fig 15C). Putrescine alone did not affect cell numbers in $Mga^{inv/inv}; CreERT2$ cells without tamoxifen, indicating that the rescue effect of exogenous putrescine is due to a deficiency in $Mga^{inv/inv}; CreERT2$ ES cells treated with tamoxifen rather than a general effect of the putrescine itself ($t = -0.152, p = 0.88$) (Fig 16A).

To ensure that the rescue effect seen in $Mga^{inv/inv}; CreERT2$ cultures was specific to putrescine, $Mga^{inv/inv}; CreERT2$ cultures were treated with the structurally related cadaverine, another cationic polyamine that has an additional carbon-link in its backbone but is not part of the polyamine synthesis pathway. Cadaverine treatment had no effect on $Mga^{inv/inv}; CreERT2$ cultures alone ($t=-0.26, p=0.80$) or on cultures that had inversion induced with tamoxifen ($t = 0.05, p = 0.96$) (Fig 16A).

To control for the possibility that the rescue effect in $Mga^{inv/inv}; CreERT2$ was a result of interaction with CreERT2 or other ES cell components, $Mga^{inv/+}$ ES cells and $Mga^{+/+}; CreERT2$ ES cells were grown and treated with the chemical combinations described above. Neither control cell lines showed any change in cell number with any of the treatments (Fig 16 B, C).
Figure 16. Box plots of cell counts of ES cell cultures treated with tamoxifen, putrescine, and cadaverine. A-C. Mga<sup>Inv/Inv</sup>; CreERT2 (A), Mga<sup>Inv/+</sup> (B), and Mga<sup>+/+</sup>; CreERT2 (C) ES cells were treated with the indicated chemicals for 48 hours before cells were counted. Scale indicates the percent of cells present compared to the average number of the untreated cells. N indicated the number individual cultures counted.
Chapter 3
Discussion

The importance of \textit{Mga} in embryonic development

We used a gene trap allele to assess the role of \textit{Mga} during development (Schnutgen et al., 2005). While this allele is not a knockout allele, we still have insight into its function. The insertion of the gene trap cassette into the 3\textsuperscript{rd} intron does not preclude transcription or translation of this allele; \(\beta\)-galactosidase activity in the embryo demonstrates that it is a functional reporter. Furthermore, the reporter appears to be expressed in the same pattern as endogenous \textit{Mga} as \(\beta\)-galactosidase activity is coincident with detection of the endogenous transcripts by RT-PCR. \textit{Mga}^{GT/GT} embryos also have a defect in tissues where \textit{Mga} transcripts are detected, notably the Epi derivatives (Yoshikawa et al., 2006). The lack of a phenotype in \textit{Mga}^{GT/+} embryos provides evidence that the mutant allele is not acting as a dominant negative in spite of the presence of the T-box in the fusion protein. We thus consider the gene trap mutant allele \textit{Mga}^{GT} to be functionally equivalent to a null allele.

Embryos with a homozygous loss of \textit{Mga} fail to develop past E4.5 with embryonic death occurring during implantation. The expression of \textit{Mga} is restricted to the ICM of embryos at E3.5 and the Epi E4.5, implicating a failure of development of this tissue in the embryonic lethality of \textit{Mga} mutants. This hypothesis is supported by blastocyst cultures, which show \textit{Mga} expression restricted to the ICM outgrowth and a failure of the ICM to expand during culture. Moreover, ES cell culture of \textit{Mga}^{Inv/Inv}; \textit{CreERT2} ES cells that had the conditional allele inverted to a mutant \textit{Mga}^{Re-inv} allele by tamoxifen formed smaller and sparser colonies with fewer total cells surviving. Because complete inversion of both alleles was not seen in any cultures, it is likely that the deficit of surviving cells is a result of completely inverted, \textit{Mga} mutant cells dying.

The loss of \textit{Mga} leads to embryonic lethality by increasing the amount of apoptosis in the embryo. Cleaved caspase 9, a marker of apoptosis, is more evident in \textit{Mga}^{GT/GT} than in \textit{Mga}^{+/+} and \textit{Mga}^{GT/+} embryos. The rise in apoptosis is not accompanied
by a change in cell proliferation in the embryo. Increased cell death of the Epi would explain both the stage of embryonic lethality as well as the presence of decidual swellings at the sites of implantation. Because the TE is initially unaffected by the loss of Mga, the decidual reaction of the uterus would still proceed, leading to decidual swellings without intact embryos. This phenotype has been noted in other mice that were mutant for ICM- or Epi-specific genes, notably Oct4 and Sox2 (Avilion et al., 2003; Nichols et al., 1998).

Because of the similar embryonic phenotype of mutants of Mga compared to mutants of genes associated with pluripotency, Oct4 and Sox2, we examined the expression of pluripotency markers at E4.5. The Epi did not appear to lose pluripotency as gauged by expression of Oct4 and Nanog. Additionally, the differentiation of PE was normal in Mga mutant embryos at E4.5 as gauged by expression of Gata4, as was differentiation of TE as judged by morphology. Together, these data demonstrate that loss of Mga does not affect the early differentiation events in the embryo.

To investigate whether pluripotency was maintained in Mga mutant embryos, we induced diapause to delay implantation. Strikingly, we found that despite entering diapause with similar numbers of pluripotent cells, the number of Nanog-expressing Epi cells in Mga mutant embryos markedly decreases during continued delayed implantation. The number of differentiated, Gata-4 positive PE cells, by contrast, initially remains stable. This suggests that the lack of Mga specifically affects the maintenance of the pluripotent cells, not the survival of differentiated cells, which do not initially require Mga to survive. Death of differentiated cells later during diapause could be a secondary effect of the lack of Epi, as has been seen in other systems (Strumpf et al., 2005). This effect is exacerbated in diapause when pluripotent cells are challenged in this way to maintain pluripotency for longer periods of time.

Because pluripotent cells were dying without Mga, we used a candidate gene approach to identify possible factors responsible this effect. We identified Odc1, the gene that codes for the polyamine synthesis enzyme ornithine decarboxylase based on the phenotypic similarity of the mouse mutants as well as the presence of E-box sites in its promoter (Bello-Fernandez et al., 1993; Pendeville et al., 2001). Because Mga has been shown to bind E-box target sites, it was reasonable to propose that loss of Mga may have an effect on Odc.
We found that ODC was reduced in the Epi of E4.5 day Mga mutant embryos. Moreover, we found that culture with exogenous putrescine, the biochemical product of ODC activity, partially rescued cell survival defects in both the Epi outgrowths of blastocyst cultures as well as those in ES cell culture. This suggests that one of the main roles of Mga in the preimplantation development of the embryos is to regulate the transcription of Odc1, an enzyme critical for the polyamine synthesis pathway. More generally, it suggests that Mga plays a crucial role in the survival of pluripotent cell populations not by regulating pluripotency itself, but rather by ensuring that cellular metabolism is functional.

**The role of polyamines in embryonic development**

Mice deficient for components of the polyamine synthesis pathway have shown that polyamines have an important role in peri-implantation development. Odc1 mutant mice fail to develop due to a defect in the growth of the ICM during implantation (Pendeville et al., 2001). Knockout of Amd1, a crucial enzyme for the production of spermine and spermidine (Fig 12A) also leads to embryonic lethality at the same time. Amd1-mutant blastocysts fail to form an ICM outgrowth in blastocyst cultures, implicating failure of ICM development as the cause of embryonic lethality (Nishimura et al., 2002) and suggesting a general role for polyamines in regulating the survival of the ICM and Epi. The importance of polyamines in preimplantation development is reflected in our own findings that embryos lacking the necessary components for the synthesis of polyamines cannot develop properly.

Synthetic disruption of Odc1 function was also found to affect pluripotent cells in vitro. Inactivation of Odc1 with DFMO in F9 teratocarcinoma cell cultures leads to a decrease in the total number of cells when counted 2 days later. Moreover, cells that did grow were differentiated, suggesting a role for Odc1 in the maintenance of pluripotent cell populations (Frostesjo et al., 1997). On the other hand, overexpression of Odc1 promoted self-renewal of ES cells in the absence of LIF. Odc1 also increased the efficiency of iPS cell generation in the absence of c-Myc (Zhao et al., 2012).
Besides ODC itself, other components of the polyamine synthesis pathway have also been linked to survival of pluripotent cell populations. Knockdown of Amd1 leads to a loss of pluripotency markers and differentiation of ES cultures. Moreover, addition of exogenous spermine to the cultures rescues this defect (Zhang et al., 2012). Together, this highlights the importance of ODC in the function of pluripotent cells, a finding that our results support.

The necessity of polyamines for peri-implantation development could be explained in a number of ways. The first is that the polyamine synthesis pathway is required to generate intermediates for other biological processes, such as DNA methylation. The methylation of cytosine, a critical step during preimplantation development, involves the incorporation of a methyl group from S-adenosyl-methionine (SAM) through the action of DNA MTase (Fig 14A). This enzyme is inhibited by high levels of decarboxylated S-adenosylmethionine (dcAdoMet), a reactant in the production of spermine and spermidine. It is possible that in the absence of putrescine, dcAdoMet accumulates and DNA methylation cannot occur. Indeed, synthetic inhibition of ODC with DFMO leads to a decrease in the amount of DNA methylation in teratocarcinoma cells (Frostesjo et al., 1997). Disruption of SAM production leading to preimplantation embryonic lethality is supported by the finding that deletion of S-adenosylhomocysteine hydrolase (Ahcy) a key synthetic enzyme for the production of SAM, leads to inhibition of ICM growth (Miller et al., 1994). Further studies to assess the DNA methylation state of Mga mutant embryos or ES cells would shed light on this possible mechanism of action.

Another possible explanation for the importance of polyamines in development is that they regulate the expression of genes important for progression through the cell cycle. Unbalancing the polyamine pool by inhibiting ODC with DFMO leads to a decrease in the expression of c-Myc in cancer cell lines. The addition of spermidine to the cultures, however, restores normal levels of c-Myc expression (Celano et al., 1988). The decrease in c-Myc leads to an increase in the amount of p21Cip1 transcription, a critical necessary for cell cycle progression (Liu et al., 2006). In the mouse model of familial adenomatous polyposis (FAP), polyamine pools are also unbalanced, though in this case through overexpression of Odc1 in the small intestine and colon. Synthetic
inhibition of ODC in this model alleviates the cancer phenotype, indicating that the proper balance of polyamines is critical for normal proliferation control (Erdman et al., 1999). Further experiments assessing the transcription of downstream genes in rescued blastocyst culture or ES cell cultures could assess whether the Mga-mediated depletion of ODC and disregulation of polyamine pools leads to a loss of control of the cell cycle. The role of Mga in the regulation of c-Myc transcription and Max network protein function in cancer could also be explored by breeding the Mga mutant allele into mouse models of cancer, specifically the APC\textsuperscript{min} mouse that models FAP or other c-Myc mutants that model cancer, such as the E\textsubscript{\mu}-myc mouse model of hematocarcinomas (Adams et al., 1985; Erdman et al., 1999).

Polyamines have also been shown to be important for regulating translation in cultured mammalian cells. HeLa cells transfected with SAT1, which codes for the catabolic protein SAT1 that is responsible for degrading polyamines by acetylating spermine and spermidine, had a decrease in the production of endogenous proteins and subsequent cell growth arrest. This decrease was not accompanied by any decrease in the synthesis of DNA or RNA indicating a post-transcriptional mechanism of gene regulation (Mandal et al., 2013). It is possible that decreasing the stores of polyamines by decreasing the expression of Odc1 would lead to a lack of translation and subsequent growth arrest during embryonic development.

A final explanation for the importance of polyamines in peri-implantation development could be that the downstream products of the polyamine synthesis pathway are needed for regulating normal cell cycle progression rather than the polyamines themselves. Genetic profiling of lymphoma samples identified Amd1 and eiF5a as common mutations leading to cancer (Scuoppo et al., 2012). These lymphomas had a deficit of hypusinated-eIF5A, a direct product of polyamine synthesis. Further experiments testing the presence of products of the polyamine synthesis pathway such as hypusine could shed light on how the loss of ODC affects the polyamine pool in mutant embryos or ES cells. Alternately, experiments using the downstream products of the polyamine synthesis pathway to rescue embryos could elucidate which products are specifically required for the peri-implantation development of the mouse embryo.
The interaction of Mga and c-Myc

The finding that Mga has a regulatory effect on the expression of Odc1 is somewhat surprising given a large body of work demonstrating that c-Myc regulates the expression of Odc1 (Bello-Fernandez et al., 1993; Pendeville et al., 2001). However, a closer examination of the Max network’s transcriptional targets provides insight into how Mga might play a role in the regulation of Odc1 or other E-box targets. It is possible, for example, that Mga and c-Myc compete for available E-box binding sites. Alternately, it is possible that Mga and c-Myc compete for available Max, the obligate heterodimerization partner that allows these genes to bind DNA.

Mga has been shown to directly compete with c-Myc for E-box binding in in vitro assays. Co-transfection of c-Myc with Max in HEK293 cells leads to an increase in transcription of a luciferase reporter driven by an E-box promoter. This effect is abrogated by co-transfection with increasing concentrations of Mga plasmid. A similar result is seen in primary rat fibroblasts, in which transfection with Mga suppresses c-Myc and Ras-mediated transformation (Hurlin et al., 1999). The interaction of Max-network members in gene regulation is seen in other systems as well. In neuroblastomas, Mga expression correlates positively with Odc1 expression. There is, however, no correlation, when samples that have normal N-myc levels rather than N-myc amplifications are considered. The fact that variable levels of N-myc can modulate the activity of Mga indicates that Max network proteins are able to regulate the activities of the entire Max network and that changing the cellular levels of any one of these components can have effects on the activities of the others (Geerts et al., 2010).

One explanation for the effects of Mga on the other Max-network proteins is competition for Max binding between proteins of the network. Because Max is necessary for bHLHZip proteins to bind DNA, only the set of bHLHZip proteins that can access Max will be active. The limited cellular stores of Max can only accommodate a finite number of interactions with other Max-network proteins, and by changing the concentration of one of the proteins, it is feasible that others can access newly available Max, or that Max homodimerization becomes favored. Max-network proteins have been
shown to alter the functions of one another when co-expressed in Cos cells (Grinberg et al., 2004), so it is a reasonable hypothesis that this is happening embryonically.

Another explanation for the effects that Mga has on putative c-Myc regulated E-box targets is the broad ability of Max-network proteins to bind the canonical E-box. Mnt, for example, has been shown to bind to promoters of multiple genes that are considered c-Myc targets during cell growth but not during quiescence, including notably Odc1 (Nilsson et al., 2004; Popov et al., 2005). This type of context dependency has been shown with other members of the Max network (Xu et al., 2001; Zervos et al., 1993). It is possible that the Odc1 promoter is regulated by Mga in the context of the peri-implantation embryo, but by other Max-network proteins in other contexts. Alternately, c-Myc may serve to amplify transcription of Odc1 after induction of transcription by Mga. This notion is supported by the fact that in human colon carcinoma cells and intestinal epithelial cells, c-Myc transcription has been shown to be dependent on ODC function. In these studies, ODC must be produced before c-Myc transcription begins; it is possible that another factor such as Mga could be responsible for initial Odc1 transcription with c-Myc amplifying transcription later (Celano et al., 1988; Liu et al., 2006). c-Myc acting as an amplifier of transcription of Odc1 rather than the as the transcriptional initiator is supported by the role that c-Myc plays in the global amplification of transcription in ES cells (Lin et al., 2012).

**A model for the role of Mga during embryonic development**

Combined with previous work on the Max network of transcription factors, our results suggest a possible mechanism for Mga to play a critical role in peri-implantation development of the embryo. Mga may interact with other proteins in the Max network, notably c-Myc, to regulate transcription of Odc1. In the absence of Mga, Odc1 is downregulated and end products of the polyamine synthesis pathway necessary for the ICM to develop are not produced in sufficient amounts. Further experiments can provide insight into the mechanism of polyamine-depletion-mediated failure of the ICM, though
loss of control of the cell cycle and subsequent apoptosis seems a likely mechanism based on the oncogenic potential of tissues that have aberrant polyamine pools.

**Future Directions**

One of the most interesting findings of our studies is the necessity of *Mga* in the survival of pluripotent tissues. While it is clear that these cells do not survive in the absence of *Mga*, whether they still retain the differentiation capacity of pluripotent cells before their death is not clear. It is possible that the cell death is a result of a loss of pluripotency. This can be addressed in a number of ways.

First, ES cells lacking *Mga* could be differentiated into embryoid bodies. Some initial work has already been done towards this aim. To ensure that tamoxifen treatment of *Mga*\textsuperscript{Inv/Inv}; *CreERT2* ES cells does not affect the differentiation of these ES cells and that the ES cells do not differentiate in the presence of residual *Mga*\textsuperscript{Inv} alleles that have not yet undergone inversion, *Mga*\textsuperscript{Re-inv/Re-inv}; *CreERT2* ES cells were generated by tamoxifen treatment of *Mga*\textsuperscript{Inv/Inv}; *CreERT2* cells in the presence of putrescine. Single colonies were picked and expanded to generate two subclones that completely lacked *Mga*\textsuperscript{inv} alleles. These ES cells are able to generate EBs in the absence of putrescine, indicating that *Mga* is not necessary for differentiation, or for survival of differentiated cells. It is unclear, though, whether these ES cells are able to survive long enough with residual putrescine from ES culture to differentiate before no longer needing putrescine as differentiated cells. More detailed studies of the differentiation of these ES cell lines that have had putrescine removed from the media at earlier time points could shed light on this issue. Furthermore, it is unclear whether the EBs formed contain differentiated tissue from all 3 embryonic germ layers. A more detailed analysis of the EBs including embryonic marker analysis could answer this question.

Another way to assess the differentiation capacity of cells lacking *Mga* is to induce differentiation in the ICM of putrescine-rescued *Mga*\textsuperscript{GT/GT} embryos in culture. Preliminary studies have been performed culturing E3.5 ICMs that were separated from the surrounding TE by immunosurgery to induce differentiation to PE. These studies have shown that without putrescine rescue, mutant ICMs are not able to differentiate into
PE while rescued ICM are, though in reduced numbers compared to $Mga^{+/+}$ or $Mga^{GT/+}$ ICMs. It is unclear whether this discrepancy is a result of fewer ICM cells surviving despite the presence of putrescine or of a deficiency in the differentiation capacity of $Mga^{GT/GT}$ ICMs. Marker or transcriptional analysis of rescued ICMs could reveal a defect in the pluripotency transcriptional program that is distinct from the defect in polyamine synthesis.

The importance of $Mga$ in the transcription of $Odc1$ also raises the possibility that other polyamine synthesis genes are also not transcribed in $Mga^{GT/GT}$ embryos. $Srm$, the gene responsible for synthesizing spermine, for example, has multiple E-box regulatory sequences in its promoter and may be regulated by $Mga$ (Forshell et al., 2010). This is supported by preliminary experiments that have shown that exogenous spermine is sufficient to rescue ICM outgrowth in $Mga^{GT/GT}$ blastocyst cultures. There are two possible explanations for this result: the first is that $Mga$ is needed to transcribe $Srm$, and that exogenous spermine is able to compensate for the lack of $Srm$ in making spermine. The second possibility is that embryos without $Mga$ are not producing spermine because of lack of ODC function and the subsequent lack of putrescine. Exogenous spermine may bypass the need for putrescine by supplying the spermine that cannot be made in the absence of putrescine. Immunofluorescence for these components could shed light on the presence or absence of other components of the polyamine synthesis pathway. Alternately, assaying the levels of polyamines could shed light on whether exogenous putrescine is being converted to spermine in $Mga^{GT/GT}$ cultures, or whether $Mga^{GT/GT}$ embryos lack components of the polyamine synthesis pathway other than ODC necessary for the production of the final polyamines.

The deregulation of polyamines and their downstream products in the progression of a number of different cancers is also interesting. As $Mga$ is able to affect the polyamine pools by regulating the expression of $Odc1$, it makes an interesting target for studies in cancer models. Specifically, the conditional $Mga^{inv}$ allele could be bred into the mouse model of human APC. This model has increased expression of $Odc1$ and its cancerous phenotype is abrogated by synthetic inhibition of ODC. According to this possible model, $Mga$ mutation would slow the progression of tumorogenesis by downregulating of $Odc1$. 
Chapter 4
Materials and Methods

Mutant alleles

Mice carrying the Mouse Genome Informatics (MGI) allele $Mga^{GT(E153E01)Wrst}$ were obtained from the German Gene Trap Consortium and alleles derived from it were used in these studies. In the following, the alleles are referred to as $Mga^{GT}$ (for gene trap), $Mga^{inv}$ (for FLP-recombinase inverted gene trap) or $Mga^{Re-inv}$ (for Cre-recombinase re-inverted gene trap). ES cell clones were isolated from E14Tg2a ES cells (Sv129P2) after retroviral infection using rsFlpRosaβgeo (FlpRBG; www.genetrap.de). The insertion of FlpRBG in intron 3 of $Mga$ was identified by splinkerette PCR (Horn et al., 2007).

Mice and genotyping

$Mga$ mutant mice were obtained from the German Gene Trap Consortium. Analysis of adult mice and embryos was performed on an random bred ICR background (Taconic Farms, Germantown, NY). Offspring were counted at weaning and ear punches or tail tips were digested in PBND lysis buffer (50 mM KCl, 10mM Tris-HCl, (pH8.3), 2.5mM MgCl2-6H2O, 0.1 mg/mL gelatin, 0.45% NP40 0.45% Tween20) with 100ug/mL Proteinase K (Roche 03115801001) for PCR genotyping. Mice carrying the $Mga^{GT}$ allele were genotyped using a three-primer PCR protocol designed to amplify wildtype and mutant bands using primers AJW360, AJW363, and AJW365. Mice carrying the $Mga^{inv}$ allele were genotyped using primers AJW360, AJW363, and AJW236. $Mga^{Re-inv/+}$ were genotyped using primers AJW360, AJW363, and AJW366. $Mga^{Re-inv/inv}$ mice were genotyped with AJW360, AJW366, and AJW236 (see table 2 in this chapter). PCR conditions were 4 minutes at 95°C, 32 cycles of 30 seconds at 95 °C, 30 seconds at 61 °C, and 40 seconds at 72°C, and 5 minutes at 72°C. PCR was performed using 2.5uM dNTPs, 1X PCR Buffer (Denville CB3702-7), 1M Betaine [Sigma B2629], 2.5uM primers, and taq that had been prepared in-house previously.

Embryos were generated and collected from timed matings using a vaginal plug as 0.5 days post coitus (defined as E0.5). After analysis, embryos were lysed in 15uL of lysis buffer (10mM Tris pH7.5, 10mM EDTA, 100mM NaCl, 0.5% Sarcosyl, 100ug/mL
Proteinase K) at 55°C for 2 hours and analyzed by genotyping using primers AJW360, AJW363, and AJW365 with Herculase II polymerase (Agilent 600675) according to manufacturer’s guidelines with 1M betaine added.

**RT-PCR**

E0.5 wild type embryos were collected from the oviduct followed by treatment with acid tyrodes solution (Sigma T1788) to remove the zona pellucida. E2.5, 3.5, and 4.5 embryos were collected by uterine flushing using M2 media (Sigma M7167-100mL). For RT-PCR, 42 E0.5 embryos, 26 E2.5, 35 E3.5, and 29 E4.5 embryos were pooled. RNA was isolated using a RNeasy mini kit (Qiagen 74104) and RT-PCR was performed using a OneStep RT-PCR kit (Qiagen 210212) as described, with primers AJW346 and AJW349 for Mga and AJW371 and AJW372 for β-actin.

**β-galactosidase activity assay**

Embryos were generated from Mga^{+/+} x Mga^{GT/+} crosses. E3.5 and E4.5 embryos were collected by uterine flushing using M2 media and E5.5, E6.5, and E7.5 embryos by isolating individual deciduae and manually dissecting embryos in cold PBS with 0.1% bovine serum albumin (Sigma A9647-50g). Embryos were fixed for 20 minutes in 4% paraformaldehyde at 4°C and then washed 3 times quickly in PBS with 0.1% Tween20 (Fischer BP337-500).

For whole mount staining, embryos were then incubated in X-Gal staining buffer (1mg/mL 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside in dimethyl sulfoxide [Sigma B4352], 20mM K₄Fe, 20mM K₃Fe, 2mM MgCl₂ in PBS) overnight at 37°C. Embryos were then washed 3 times in PBS with 0.1% Tween and fixed in 4% paraformaldehyde before being photographed.

For cryosectioning, embryos were transferred to 20% sucrose overnight and then embedded in O.C.T. Compound (Tissue-Tek 4583). 10-12µm sections were cut and dried and slides were fixed in 4% paraformaldehyde for 10 minutes at 4°C and incubated in X-Gal staining buffer overnight at 37°C. Slides were fixed in 4% paraformaldehyde and then counterstained with Eosin Y (Sigma 318906-500mL) and mounted in Permount (Fisher SP15-500) before photographing.
For greater sensitivity, Salmon-Gal was used to confirm expression patterns (Sundararajan et al., 2012). Embryos were flushed with M2 and fixed briefly at 4C with 4% PFA. Embryos were washed twice with S-Gal rinse solution (0.1% sodium deoxycholate, 0.2% IGEPAL (Sigma I8896), 2mM MgCl$_2$ and 0.1 M phosphate buffer pH 7.3). Embryos were then incubated with 1mg/ml Salmon Gal (6-chloro-3-indolyl-B-D-galactopyranoside, Lab Scientific X668) and 6µg/ml NBT (4-nitro blue tetrazolium chloride, Sigma N6876) dissolved in 70% N, N-dimethylformamide in water at 37C for 1 or more days.

Blastocyst outgrowth in vitro

Embryos were generated from Mga$^{GT/+}$ x Mga$^{GT/+}$ crosses and collected at E3.5 by uterine flushing using M2 media. Zona pellucidae were removed by incubation in acid tyrodes solution for approximately 2 minutes at room temperature. Embryos were then plated on tissue culture dishes (BD Falcon 353003) in 20µL drop of ES media (DMEM [Gibco 11965-092], 13% FBS [Hyclone Cat #SH30071.03 Lot #ARG27092], 1% Pen/Strep [Gibco 15070], 1% GlutaMax [Gibco 35050-061], 1% Sodium Pyruvate [Gibco 11360-070], 1% Non-Essential Amino Acids [Gibco 11140-050], 0.1% Beta-Mercaptoethanol [Gibco 21985-023], LIF derived from CHO cells in-house) and covered in mineral oil (Fischer 0122-1). Embryos were cultured at 37°C in 5% CO$_2$ in air (Bhatnagar et al., 1995). Cultures were photographed daily and the surface area of the ICM and trophoblast was assessed by morphology and quantitated using ImageJ (NIH, http://rsb.info.nih.gov/ij/).

For rescue experiments, embryos were cultured in ES media with 200uM putrescine (Sigma P5780-5g) dissolved in water. Following culture, embryos were scraped off of the dish and genotyped.

Immunohistochemistry

Embryos were generated from Mga$^{GT/+}$ x Mga$^{GT/+}$ crosses and collected by uterine flushing using M2 media. Immunohistochemistry was performed as previously described (Artus et al., 2010). Briefly, embryos were cultured in DMEM/HEPES (Gibco 12430-054) and 10% FBS (Hyclone Cat #SH30071.03 Lot #ARG27092) for 20 minutes at 37°C
and 5% CO\textsubscript{2} and then fixed in 4% paraformaldehyde with 0.1% Tween20 and 0.01% TritonX (Fischer BP151-500) for 10 minutes at room temperature or overnight at 4C. Embryos were then washed in PBT (PBS with 0.1% TritonX) before permeabilization with 0.5% TritonX for 20 minutes at room temperature. After 3x 5 minute washes with PBT, antigens were unmasked with NH\textsubscript{4}Cl (Sigma A-4514) in PBT for 10 minutes at room temperature. Following 2x 5 minutes washes, embryos were blocked in 2% donkey serum in PBT for 45 minutes at room temperature. Embryos were then incubated with the first primary antibodies overnight at 4\textdegree C in 2% donkey serum. The following day, embryos were washed 3 times with PBT and incubated with the second primary antibody overnight at 4\textdegree C in 2% donkey serum. The following day, embryos were washed 3 times with PBT and incubated with secondary antibodies overnight at 4\textdegree C in the dark. Embryos were again washed 3 times with PBT and incubated with Hoecst 33342 (Sigma B2261) diluted 1:500 overnight at 4\textdegree C in the dark. Embryos were washed 2 more times and then analyzed using a Nikon A1R confocal microscope and NIS Elements v4.0 software with MatTek 35mm petri dishes with coverglass (MatTek P35G-0-10-C).

Embryos were then lysed and genotyped with Herculase II polymerase. Statistics were analyzed using Fisher’s exact probability test.

**Embryonic diapause and immunosurgery**

Diapause was induced in pregnant females at E2.5 by subcutaneous injection of 3mg Depo Provera suspended in PBS (Medroxyprogesterone 17-acetate Sigma M1629-1g) and intraperitoneal injection of 20\textmu g tamoxifen in sunflower seed oil (Sigma T5648-1g). Embryos were then flushed from the uterus with M2 1-7 days later.

To perform immunosurgery, embryos were washed twice with Hams F12 media (Gibco 11765-054) and incubated in 20% rabbit anti-mouse serum antibody (Sigma M5774) in Hams F12 for 30 minutes at 37\textdegree C in 7% CO\textsubscript{2} in air. Embryos were washed 3 times in Hams F12 before incubation with 20% guinea pig compliment (CalBiochem 234395) in Hams F12 with 1:100 Propidium Iodide (Sigma P4864) and 1:100 Hoechst 33342. Embryos were washed quickly 3 times in Hams F12 and squashed on a glass slide with a glass coverslip before being photographed on a Nikon fluorescent microscope with NIS Elements software.
Immunohistochemistry was performed as described, though the embryos remained intact allowing PCR genotyping after imaging. Statistics were analyzed using Fisher’s exact probability test and Mann-Whitney U test.

**ES Cells**

$Mga^{inv/inv}; CreERT2$ and $Mga^{inv/+}$ ES cell lines were derived using previously established protocols (Batlle-Morera et al., 2008). Embryos were generated from $Mga^{inv/inv}; CreERT2 \times Mga^{inv/+}; CreERT2$ matings and diapause was induced at E2.5. Embryos were flushed with M2 4 days after diapause induction. Immunosurgery was performed as described and isolated ICMs were plated on gelatin-coated tissue culture dishes in mES media supplemented with recombinant human BMP4 (R&D Systems 314-BP-010) and MEK inhibitor PD98059 (Cell Signalling Technology 9900S). After 7-10 days, ICM outgrowths were trypsinized with 0.25% trypsin (Gibco 25200-056) and replated on mitomycin-C (Sigma M4287) treated MEFs. Inversion was induced using 4-hydroxytamoxifen (Sigma H6278). “Inverted” cultures were treated with 200µM putrescine and 200µM cadaverine (Sigma D22606) dissolved in water. For cell count assays, cells were counted and then plated in separate wells, allowed, to attach overnight, and then treated the next day. After 48 hours of chemical treatment, cells were trypsinized and counted on a hemocytometer in duplicate or triplicate and the counts averaged. Statistics were analyzed using a Student’s t-test.
Table 1 – Primer sequences

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Table 2 – Primer combinations for genotyping

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References


Chapman, D. L., Garvey, N., Hancock, S., Alexiou, M., Agulnik, S. I., Gibson-Brown, J. J., Cebra-Thomas, J., Bollag, R. J., Silver, L. M. and Papaioannou,


Appendix

Additional publications produced in the course of doctoral research:


PATTERNS & PHENOTYPES

Expression of Slit and Robo Genes in the Developing Mouse Heart

Caroline Medioni,† Nicolas Bertrand,† Karim Mesbah, Bruno Hudry, Laurent Dupays, Orit Wolstein, Andrew J. Washkowitz, Virginia E. Papaioannou, Timothy J. Mohun, Richard P. Harvey, and Stéphane Zaffran*

Development of the mammalian heart is mediated by complex interactions between myocardial, endocardial, and neural crest-derived cells. Studies in Drosophila have shown that the Slit-Robo signaling pathway controls cardiac cell shape changes and lumen formation of the heart tube. Here, we demonstrate by in situ hybridization that multiple Slit ligands and Robo receptors are expressed in the developing mouse heart. Slit3 is the predominant ligand transcribed in the early mouse heart and is expressed in the ventral wall of the linear heart tube and subsequently in chamber but not in atrioventricular canal myocardium. Furthermore, we identify that the homeobox gene Nkx2-5 is required for early ventral restriction of Slit3 and that the T-box transcription factor Tbx2 mediates repression of Slit3 in nonchamber myocardium. Our results suggest that patterned Slit-Robo signaling may contribute to the control of oriented cell growth during chamber morphogenesis of the mammalian heart.


Key words: Slit/Robo pathway; cardiac development; mouse; Tbx; atrioventricular canal

INTRODUCTION

Cardiogenesis is one of the earliest and most critical steps during vertebrate organogenesis. Heart development begins when cardiac progenitor cells in the anterior lateral mesoderm cluster in the primary heart field (Harvey, 2002). These cells give rise to the cardiac crescent and linear heart tube containing the future left ventricle and atrioventricular canal (AVC; see Buckingham et al., 2005). Subsequently the heart tube undergoes rightward looping (Harvey, 2002). As looping progresses, cells of the second heart field in splanchnic mesoderm are added to the heart tube to form the outflow tract (OFT), right ventricle, atria and inflow tract regions (Buckingham et al., 2005). Subsequently, atrial and ventricular chambers form through a localized process that involves differential growth or “ballooning” of the outer curvature of the heart tube (Christoffels et al., 2000). Importantly, part of the heart tube, including the OFT, inner curvature, AVC, and inflow tract, escapes this developmental chamber program through the repressive action of the T-box factors, Tbx5 and Tbx3 (Habets et al., 2002; Christoffels et al., 2004b; Harrelson et al., 2004; Bakker et al., 2008). Regionalized gene expression provides evidence for the presence of additional Supporting Information may be found in the online version of this article.

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8Grant sponsor: Agence Nationale pour la Recherche, Grant number: ANR-06-MRAR-003; Grant sponsor: NIH; Grant number: 5R37HD033082; Grant sponsor: The European Commission, Grant number: HEALTH-2007-B-223463.
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of dorsoventral patterning in the early tube that precedes chamber development (Christoffels et al., 2004a). A retrospective clonal analysis of cardiac cells has shown that myocardium, from the time of its formation, is a polarized and regionalized tissue in which oriented cell growth may be important in shaping the chambers (Meiha et al., 2004). Although key factors that play a role in forming the heart tube have been identified, including GATA4, NIK2-2, dHAND, Bmp5, or Raldh2 (Harvey, 2002), the molecular effectors of cell polarity and cell shape changes remain unknown.

The extracellular-matrix molecule Slit and its Robo (roundabout) family receptors have been implicated in the regulation of cell polarity and morphogenesis during formation of the cardiac tube in Drosophila (Qian et al., 2005; MacMullin and Jacobs, 2006; Medioni et al., 2008; Santiago-Martinez et al., 2008). In particular, the Slit-Robo pathway is required for progressive polarization of cardiac cells during migration to the midline (Meiha et al., 2008). In contrast to the single Slit and three Robo genes in Drosophila, three distinct Slit genes (Slit1, Slit2, and Slit3) have been identified in mammals (Chedotal, 2007). Slit functions as a repulsive ligand for the Robo-family receptors in the central nervous system (CNS), and acts both attractively and repulsively in somatic muscles (Kidd et al., 1998, 1999; Brose et al., 1999; Simpson et al., 2000; Wu et al., 2001). In addition, both gene families display distinct expression patterns outside the CNS (Holmes et al., 1998; Yuan et al., 1999; Strickland et al., 2006). Remarkably, Slit2 is widely expressed in different organs, including the tongue, kidney, pharynx, umbilical cord vein, heart, lung, and diaphragm (Yuan et al., 1999; Liu et al., 2003; Yuan et al., 2003). Consistent with its expression in non-neural tissues, studies have established that Slit3 is required for angiogenesis and formation of the diaphragm and kidney (Liu et al., 2003; Yuan et al., 2003; Zhang et al., 2009).

The role of the Drosophila Slit-Robo pathway in regulating changes in cell shape during cardiac tube formation prompted us to conduct a detailed analysis of murine Slit and Robo gene expression during mouse heart development. We describe that Slit3 in particular shows a specific localization in the ventral region of the forming heart tube and subsequently restricts to chamber myocardium. We depict in J) showing expression of Slit3 in the linear heart tube. In addition, we demonstrate that the T-box factors Tbx2 and Tbx20 mediate the restriction of Slit3 expression to the chamber myocardium. Given the spatial and temporal expression profile of Slit3 and its role in polarized growth and migration in other tissues, we propose that Slit/Robo signaling may be required for cardiac chamber expansion.

RESULTS AND DISCUSSION
Expression of Mouse Slit and Robo Genes During Heart Development
Previous studies in Drosophila have shown that the Slit-Robo signaling pathway controls cardiac cell polarity and formation of the cardiac lumen (Medioni et al., 2008). Therefore, we decided to analyze the expression pattern of murine Slit and Robo genes in the developing heart. In contrast to other tissues, detailed analysis of the cardiac expression pattern of Slit and Robo genes has not been reported yet. Holmes et al., 1998; Yuan et al., 1999; Holmes and Niwander, 2001; Liu et al., 2003; Yuan et al., 2003). Mouse embryos between 7.5 and 12.5 days of development (E7.5–E12.5) were used with antisense riboprobes for Slit1, Slit2, Slit3, Robo1, Robo2, Rig1, Robo3, and Robo4 (Figs. 1, 2; also see Supp. Fig. S1, which is available online).

Fig. 1 Expression pattern of Slit genes during embryonic heart development. Whole-mount in situ hybridization analysis of embryos with Slit1 (A–D), Slit2 (E–H), and Slit3 (I–L) probes. A: Ventral view of embryonic day (E) 8.5 embryo showing Slit1 expression in the ventral midline (arrowhead). Slit1 is not detected in the forming heart tube. B: Lateral view of E9 embryo showing Slit1 expression in the roof plate (arrowhead), in the floor plate (double arrowheads), and in the pharyngeal region (white arrow). C: Frontal section (same embryo depicted in B) showing Slit1 expression in the mesodermal core of the pharyngeal arch (white arrow) and in the floor plate of the spinal cord. D: High magnification picture of heart at late E9.5 showing Slit1 in atrial wall. D: Dissected E12.5 heart showing no expression of Slit1. E: In situ hybridization with Slit1 on section of E12.5 heart. F: Slit2 was not detected in the heart of embryo at E8.5 stage. Note its expression at the ventral midline (arrowhead). G: Lateral view of early E9.5 embryo showing Slit2 expression in the pharyngeal region (asterisk). Slit2 is also detected in the roof plate (arrowhead), the notochord (double arrowheads) and the somites (white arrowhead). H: Frontal section (same embryo depicted in F) showing Slit2 expression in the pharyngeal ectoderm surface (arrow). I: Dissected E9.5 heart showing no expression of Slit2. J: A robust expression of Slit2 is detected in trabecula of both ventricles at E12.5. K: In situ hybridization on section of E12.5 heart shows expression in the trabeculae (arrowhead). L: Ventral views of E7.5 and E8.5 embryos showing Slit2 expression in the cardiac crescent and the forming heart tube. Expression of Slit2 is also detected in the ventral midline (black arrowhead) and the somites (white arrowhead) of E8.5 embryos. M: Lateral view of early E9.5 embryo showing Slit2 expression in the heart and the pharyngeal ectoderm surface (asterisk) and in the somites (white arrowhead). N: Frontal section (same embryo depicted in J) showing expression of Slit2 in pharyngeal ectoderm surface (arrow). O: High magnification picture of heart at late E9.5. Expression of Slit3 is seen in the outflow tract, the atria and ventricles of the embryonic heart. P: At E12.5, expression of Slit3 is maintained in the right and left atria and in great arteries, the aorta and pulmonary trunk. Q: Section of the heart shown in L, ao, aortic; cc, cardiac crescent; ht, heart tube; la, left atrium; lv, left ventricle; oft, outflow tract; pt, pulmonary trunk; ra, right atrium; rv, right ventricle.

Fig. 2 Expression pattern of Robo genes during embryonic heart development. Whole-mount in situ hybridization analysis of embryos with Robo1 (A–D) and Robo2 (E–H) probes. A: Ventral view of early embryonic day (E) 8.5 embryo showing Robo1 expression in the ventral pole (white arrows) of the forming heart tube. B: Lateral view of early E9 embryo. Robo1 expression is detected in the notochord (double arrowhead), the somites (white arrowhead), and the ectodermal pouches of the pharyngeal arches (asterisk) but not in the heart. C: High magnification picture of the heart at late E9.5 showing Robo1 in the cushions (asterisk) of the outflow tract. D: Dissected E12.5 heart showing expression of Robo1 at the base of the great arteries (arrow). E: Ventral view of early E8.5 embryo. High Robo2 expression levels are visible in the neural tube and the ventral pole (white arrows) of the forming heart tube. F: Lateral view of E9.5 embryo. Weak expression of Robo2 is detected in the looped heart, the ectoderm of the pharyngeal arches (asterisk) and the notochord (double arrowheads). G: High magnification of the heart at E10.5 showing Robo2 in both atria. H: Dissected E12.5 heart showing expression of Robo2 in the great arteries (arrowheads), ht, heart tube; la, left atrium; lv, left ventricle; oft, outflow tract; ra, right atrium; rv, right ventricle.
Fig. 1.

SLIT AND ROBO EXPRESSION IN MOUSE HEART

Fig. 2.
During this period of development, Slit2 expression was primarily observed in the roof plate and the floor plate (Fig. 1A–B). Slit1 transcripts were also observed in the mesodermal core of the pharyngeal arches (Fig. 1H, I). Interestingly, pharyngeal mesoderm has been shown to contribute to the formation of the OPT as well as the pharyngeal arch artery (PAa) development (see Kelly and Buckingham, 2002). At E9.5, Slit1 expression was detected in the developing heart in the left and right atria (Fig. 1C). However, at E12.5 we did not observe any expression in the heart (Fig. 1D, E).

Similarly to Slit1, Slit2 expression was observed prominently in neural tissue (Fig. 1E, F). From E8.5 to E9.5, Slit2 expression was not detected in the myocardium; however, it was highly expressed in the pharyngeal region at these stages (Fig. 1F) as reported by others (Yuan et al., 1999; Calmont et al., 2009). Strong expression of Slit2 was seen in the pharyngeal surface ectoderm (Fig. 1F). This tissue has been shown to be a crucial source of signals for fourth PAA formation and remodeling (Kirby, 2007). Slit2 has also been identified as a downstream target of Tbx1, and is implicated in cardiac neural crest cells (NCC) migration at the time of PAA formation (Calmont et al., 2009). While no clear expression in the embryonic heart was detected at E9.5 (Fig. 1G), a strong expression of Slit2 was observed in the trabecular region of the ventricular chambers at E12.5 (Fig. 1H, I). Of interest, the trabecular formation occurs when cardiomyocytes migrate toward the endocardium, which is coincident with up-regulation of cell adhesion molecules (Ong et al., 1998). Thus, our observation suggests that other cell signaling molecules such as Slit may be involved in this process.

Slit3 is the earliest Slit gene to be expressed in the developing heart. Transcripts were observed in the cardiac crescent at E7.5 and in the linear heart tube at E8.5 (Fig. 1J). At E8.5, Slit3 expression is observed on the ventral wall of the linear heart tube (see Fig. 3A, B). Slit3 is also expressed in the ventral midline and developing somites (Fig. 1J). Unlike the other Slit genes, Slit3 expression was observed in all compartments of embryonic heart at E9.5, restricted to the outer curvature of the looped heart (Fig. 1J, K). The myocardium of the outer curvature is known to give rise to the ventricular chamber or "working" myocardium (de la Cruz and Markwald, 1999; Christoffels et al., 2000). Analysis of the distribution of closely related myocyes has demonstrated that different patterns of oriented cell growth underlie regional differences in morphogenesis within the embryonic heart (Meilhac et al., 2004). The expression pattern of Slit3 and its established role in polarized growth and migration in other tissues suggest implication of Slit-Robo signaling in the oriented cell growth that accompanies ballooning of the ventricular chambers (Christoffels et al., 2004a). By E12.5, expression of Slit3 was observed only in myocardium of the atria and at the base of the great arteries (Fig. 1J, L), in agreement with published expression data (Liu et al., 2003). Remarkably, expression of Slit2 and Slit3 in ventricular chambers seems to be complementary in the endocardium and myocardium respectively. This observation suggests requirement of a specific Slit ligand expression combinatorial to the expression of the basic helix-loop-helix (bHLH) transcription factor gene, Hand1, which is restricted to the ventral wall of the forming heart tube (Fig. 3A–D; Biben and Harvey, 1997; Christoffels et al., 2000; Togi et al., 2004a). Although Slit3 is expressed at high levels in the developing heart, early cardiac defects have not been reported in Slit3 mutant embryos (Liu et al., 2003; Yuan et al., 2001). However, the expression of Slit3 detected in the outer curvature of the looped heart and the enlarged right ventricle observed in the hearts of Slit3 mutant mice (Liu et al., 2003), suggest a role for Slit signaling in ventricular chamber formation. Further studies are required to determine whether subtle changes may exist in the heart of these mutants especially during the formation of the myocardium chambers and the great arteries.

We subsequently examined expression of Slit3 in mutant mice affecting cardiac morphogenesis. Mutations in the NK-like homeobox gene, Nkx2-5/Csx, cause early embryonic lethality with cardiac development arrested at...
the linear heart tube stage, before looping (Komuro and Izumo, 1993; Lyons et al., 1995; Biben and Harvey, 1997). In Nkx2.5−/− embryos, the early expression of Slit3 was indistinguishable from that in control embryos (Fig. 3E). However, we found that the dorsoventral pattern of Slit3 expression in the linear heart tube was perturbed and expression was observed throughout the mutant heart tube (Fig. 3E,F). This result suggests that the myocardium of Nkx2.5−/− embryos is competent to express Slit3 but not to interpret signals that restrict expression on the ventral side of the heart. Of note, two putative Nkx2-5 binding motifs (TGAAGTGATG and TAAAGTGGGT) are found in a 3,000 bp Slit3 5′ proximal fragment as predicted using the TFSEARCH program (http://mbs.cbrj.jp/research/db/TFSEARCH.html).

Expression of Robo receptor genes in the venous pole (Fig. 2A,E) incited us to examine their expression in Nkx2-5−/− embryos. Of interest, we did not detect expression of Robo2 in the linear heart tube of Nkx2-5−/− mutant embryos (Supp. Fig. S2). This observation suggests that Nkx2-5 regulates in a different way Slit ligand and Robo receptor genes during the formation of the embryonic heart tube.

Fig. 3. Dorsoventral patterning of Slit3 expression in the linear heart tube requires Nkx2-5. A: Ventral view of embryo at early embryonic day (E) 8.5. Slit3 is highly expressed in the ventral side of the forming heart tube. B: Section (same embryo as depicted in A) showing that Slit3 expression in the heart tube is strictly ventral as delimited by the arrowheads. C: Expression of Hand1 is shown as a reference to indicate the ventral side (arrowheads) of the heart tube at E8.5. D,E: Comparison of Slit3 expression in wild-type (WT) and Nkx2-5−/− embryos at E8.5. D: Lateral view of WT embryo showing high expression of Slit3 in the ventral side of the forming heart tube. D: Section of the embryo shown in D. Note the expression of Slit3 in the floor plate (arrow). E: Slit3 expression is maintained in Nkx2-5−/− embryo. F: Section (same embryo as shown in E) reveals that Slit3 is uniformly expressed in heart of Nkx2-5−/− embryo. ht, heart tube; nt, neural tube.

Fig. 4. Dynamic expression pattern of Slit3 during heart development. In situ hybridization (A–E) and immunofluorescence (F) were used to detect spatial expression of Slit3 in hearts from embryonic day (E) 9 to E10.5. A,B: Expression of Slit3 is detected in the whole heart of E9 embryo, whereas it is downregulated in the AVc of late E9.5 embryo. C: Section (same embryo as depicted in B) showing expression of Slit3 in the chambers but not in the AVc. Note weak expression of Slit3 in the trabeculae of the left ventricle. D: Lateral view of E10.5 embryo. High expression of Slit3 is detected in the left atrium. Note Slit3 expression in the limb buds and the dermomyotome (white arrowhead). E: Dissected heart from the embryo shown in D. High expression is observed in the right and left atria and the outflow tract. F: Expression of Slit3 protein (red) is detected in the ventricular myocardium and trabeculae but not in the AVc of heart at E10.5. Higher magnification of the trabeculae region delimited by the dotted line is shown in the inset. F1: Immunodetection of Slit3 (red) and DAPI (blue) staining. Note Slit3 expression in the cytoplasm, ao, aorta; avc, atrioventricular canal; la, left atrium; lb, limb bud; lv, left ventricle; of, outflow tract; pt, pulmonary trunk; ra, right atrium; rv, right ventricle.
Regulation of the Chamber-Specific Expression Profile of Slit3

In looped hearts (E9–E9.5), Slit3 expression was confined to the atrial and ventricular myocardium but was clearly absent from the AVC (Fig. 4A–C), a pattern resembling that of atrial natriuretic factor (ANF; see Supp. Fig. S1A). Slit3 protein expression was restricted to the atria and the OPT (Figs. 1L, 4C–E). Immunohistochemistry revealed Slit3 protein expression in both atrial and ventricular myocardium at E10.5 (Fig. 4F). The AVC region was negative, confirming our results in situ hybridization. Detection of Slit3 protein but not mRNA in the ventricles of the heart at E10.5 (Fig. 4E,F) indicates a persistence of the protein in the chamber myocardium. Although Slit3 expression overlaps with Robo1 and Robo2 in the venous pole and later only with Robo2 in the atria, no Robo receptor genes were detected in the ventricular myocardium of the heart (Fig. 2). Despite its closest link with Robo receptors, Slit contains domains that suggest association with the extracellular-matrix receptors (Chedotal, 2007). Furthermore, recent studies in Drosophila have proposed that Slit is localized on cardiac cells by association with Dystroglycan (Dg), a proteoglycan (Medioni et al., 2008), and possibly also with pPSI/pPS1 Integrin (MacMullin and Jacobs, 2006).

The absence of Slit3 gene expression and protein in AVC myocardium suggested potential regulation by the T-box transcriptional repressors Tbx2 and Tbx3 that are restricted to non-chamber myocardium (Supp. Fig. S3LC), where they repress the chamber transcriptional program (Habets et al., 2002; Christofels et al., 2004b; Harrelson et al., 2004; Bakker et al., 2006; Mesbah et al., 2008). Therefore, we examined Slit3 expression in mutant embryos deficient for Tbx2 and Tbx3. In situ hybridization on stage-matched embryos revealed activation of Slit3 in the AVC of E10.5 Tbx2−/− but not Tbx3−/− hearts (Supp. Fig. S4L). This observation indicates that Tbx2 alone is sufficient to repress Slit3 in the nonchamber myocardium of Tbx3−/− hearts, consistent with previous findings on other chamber-specific genes (Bakker et al., 2006; Mesbah et al., 2008). The T-box factor Tbx20 is essential for embryonic chamber formation through its negative regulation of Tbx2 in the myocardium and endocardium (Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005; Takeuchi et al., 2005). To test the hypothesis that ectopic Tbx2 expression may be able to repress Slit3 in the forming heart, we analyzed Slit3 expression in Tbx20−/− embryos. Mutant embryos showed severe cardiac abnormalities including rudimentary ventricular chambers that did not further differentiate (Stennard et al., 2005). We could not detect any Slit3 expression in Tbx20−/− hearts at E9 (compare Fig. 5B,E with 5A,A'). Of interest, Drosopha Slit is also perturbed in the embryonic heart tube of Tbx20-ortholog (H15omin) mutants (Qian et al., 2005), suggesting a high evolutionary conservation of this regulation pathway. We also examined Slit3 expression in embryos misexpressing Tbx2 throughout the embryonic heart under the control of the XMLC2 promoter (XMLC2-rtTA/tetO-Tbx2) (Dupays et al., 2009). Consistent with our observation in Tbx20−/− embryos, Slit3 expression was significantly reduced (Fig. 5C,C'). The residual weak expression of Slit3 observed in these embryos may be explained by the mosaic expression of the transgene at this stage (Dupays et al., 2009). Moreover, we found three conserved T-box binding elements (TBE) in Slit3 (Kispert and Herrmann, 1993; Sinha et al., 2006) that are present in the Slit3 5′ proximal promoter (Fig. 4D). The requirement of these TBEs for repression in the AVC and the complementary expression pattern between Tbx2 (Supp. Fig. S3) and Slit3 (Fig. 4B) prompted us to study the interaction of Tbx2 with the TBE motifs identified in the Slit3 promoter using electrophoretic mobility shift assay (EMSA) experiments. Oligonucleotide probes corresponding to the three TBE motifs were used. Tbx2 bound to the wild-type TBE2 motif but not TBE1 and TBE3, because a shifted band was detected only in lanes 11 and 12 (Fig. 4E). However, when the EMSA was performed with a mutated TBE2 motif this binding was abolished (Fig. 4E). Together these results suggest that Slit3 expression may be directly repressed by Tbx2 during AVC formation.

Conclusion

In this study, we have characterized Slit ligand and Robo receptor gene expression in the developing mouse heart. Our results suggest that Slit-Robo signaling, essential for morphogenesis of the Drosophila heart tube, may play roles in oriented cell growth during atrial and ventricular morphogenesis in vertebrates. Furthermore, we identify two upstream regulators of Slit3 expression, the predominant Slit ligand expressed in the early mouse heart: analysis of Slit3 expression in different mutant mouse embryos reveals that Slit3 is repressed to the ventral wall of the linear heart tube by Nkx2-5 regulated mechanisms and excluded from AVC myocardium by the transcriptional repressor Tbx2.

EXPERIMENTAL PROCEDURES

Animals and Tissue Preparation

All experiments involving animals were performed in accordance with French guidelines on the care and use of laboratory animals. After death by CO2 asphyxiation, embryos were removed from timed-pregnant CD1 or mutant mice. The day of vaginal plugging was defined as 0.5. Embryos were genotyped by polymerase chain reaction (PCR) using genomic DNA isolated from yolk sacs. The null alleles Tbx20tm20, Tbx30tm1Pa, Tbx20tm2, and Nkx2-5tm1Pa were maintained on a mixed genetic background (Bihen et al., 2006; Davenport et al., 2003; Harrelson et al., 2004; Stennard et al., 2005). Somites were counted for developmental staging and a sample of the yolk sac was taken for PCR genotyping using the following primers.

Tbx2: the primers 5′-GGC CTC AAG TAG CCT GGA A-3′, 5′-AGG CCA
ACA GAA GAG CAG A-3', and 5'-CTA AGG GAA CAT AAT GAG G-3', result in a 350 bp wild-type band and a 500 bp mutant band. Tbx2: the primers 5'-CCA GCC AGG GAA CAT AAT GAG G-3', 5'-CTG TCC CCT GGC ATT TCT GG-3', and 5'-CCT GCA GGA ATT CCT CGA CC-3' result in a 180 bp wild-type band and a 88 bp mutant band.

Nkx2-5: the primers 5'-GAA CCT GGA GCA GCA GCA GCG TAG C-3' and 5'-CAG AAG GGA AGA GCT TGA GGT TCT C-3' result in a 308 bp wild-type band and a 1,376 bp mutant band.

The tetO-Tbx2 and xMlc2-rtTA transgenes have been previously described (Dupays et al., 2009). Dose-cycline was administered to pregnant females either by intraperitoneal injection (2 mg of Dox in 0.5 ml of 0.9% aqueous NaCl) at the indicated stage or by means of food (2 mg of Dox in 0.5 ml of 0.9% aqueous NaCl) from stages specified.

For early developmental stages whole embryos were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight at 4°C, dehydrated and kept in methanol. Hearts or trunks were dissected and fixed in 4% PFA in PBS overnight at 4°C, transferred to 15% sucrose in PBS, followed by 15% sucrose 7% gelatin, and frozen in liquid nitrogen before cryo-sectioning at 10 μm.

**Whole-Mount In Situ Hybridization**

Whole-mount in situ hybridization was carried out as published (Zaffran et al., 2004). Probes were labeled according to the manufacturer's instruction using the digoxigenin (DIG) -RNA labeling mix (Roche). The probes used for in situ hybridization were mSlit2 and mSlit3 (Yuan et al., 1999), rat Slit1, Robo1, Robo2 (Kidd et al., 1998), Rig1/Robo3, and mRobo4 3'-untranslated region.
DNA-Binding Assay

For EMSA, the Tbx2 protein was produced with the TNT (T7) coupled in vitro transcription/translation system (Promega). Production yields of Tbx2 protein was estimated by \(^35\)S]methionine labeling. EMSAs were performed in a 20-μl volume on ice with 1000 cpm (0.5 ng) of either probes. Probes used were double-stranded: TBE1 (5'-TTTTTTGTGTATGAGATGCA), TBE1mut (5'-TATGGCGCGCGCTCCACCG), TBE2mut (5'-TATGCGTTTACCGTACCGC), TBE2 (5'-TATGGCGCGCGCTCCACCG), TBE3 (5'-TATGCGTTTACCGTACCGC) and TBE3mut (5'-TATGCGTTTACCGTACCGC) from the Tbx2 promoter. Briefly, 3 or 9 μl of Tbx2 protein was gently added and incubated for 30 min with labeled probes and 0.1 mg of non-specific competitor poly(dC) in a binding buffer 5% composition of 20% glycerol, 50 mM Tris-HCl pH7.5, 250 mM NaCl, 2.5 mM ethylenediaminetetra-acetic acid, 2.5 mM dithiothreitol, and 0.25 mg BSA then loaded on a 4% polyacrylamide gel in 0.25× TBE buffer. The gel was dried and analyzed with a PhosphorImager.

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We thank Dr. David Ornitz for his kind and generous gift of the Slit2 and Slit3 vectors, and Dr. Alain Chedotal for providing the Slit1 and Robo1, Robo2, and Robo3 vectors. We thank Dr. Fugia Murakami for generously providing Robo1 and Robo2 antibodies. We express our gratitude to Dr. Florence Besse for allowing Caroline Medioni to complete this work during her post-doc in her lab. We are grateful to Dr. Samir Merabet for allowing Bruno Hudry to carry out part of this work. We thank Dr. Robert Kelly for discussions and comments on the manuscript.

REFERENCES


Developmental Dynamics


Focus Article

Diverse functional networks of Tbx3 in development and disease

Andrew J. Washkowitz, Svetlana Gavrilov, Salma Begum and Virginia E. Papaioannou

The T-box transcription factor Tbx3 plays multiple roles in normal development and disease. In order to function in different tissues and on different target genes, Tbx3 binds transcription factors or other cofactors specific to temporal or spatial locations. Examining the development of the mammary gland, limbs, and heart as well as the biology of stem cells and cancer provides insights into the diverse and common functions that Tbx3 can perform. By either repressing or activating transcription of target genes in a context-dependent manner, Tbx3 is able to modulate differentiation of immature progenitor cells, control the rate of cell proliferation, and mediate cellular signaling pathways. Because the direct regulators of these cellular processes are highly context-dependent, it is essential that Tbx3 has the flexibility to regulate transcription of a large group of targets, but only become active on a small cohort of them at any given time or place. Moreover, Tbx3 must be responsive to the variety of different upstream factors that are present in different tissues. Only by understanding the network of genes, proteins, and molecules with which Tbx3 interacts can we hope to understand the role that Tbx3 plays in normal development and how its aberrant expression can lead to disease. Because of its myriad functions in disparate developmental and disease contexts, Tbx3 is an ideal candidate for a systems-based approach to genetic function and interaction. © 2012 Wiley Periodicals, Inc.

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INTRODUCTION

The T-box family of genes is an ancient and evolutionarily conserved group of transcription factor genes defined by their DNA-binding domain, known as the T-box. First discovered in mouse, the T-box family derives its name from the mesoderm-specification gene Brachyury (T). Each T-box factor binds a specific core sequence, the T-half-site, found in the promoters of target genes, often in tandem or in different orientations. These T-half sites are accompanied by other transcription factor binding sites, giving them specificity. It is the interactions with these other transcription factors that allow T-box genes to play a variety of roles during disparate development processes.

The 17 members of the T-box gene family in mouse have been grouped into five subfamilies based on sequence similarity. Tbx3 is a member of the Tbx2 subfamily, a group that also includes Tbx2, Tbx4, and Tbx5. This subfamily arose during a tandem duplication event followed by chromosomal duplication and dispersion. Tbx3 and Tbx2 are closely related members sharing 90% amino acid identity in the T-box and having many overlapping areas of expression.

During normal mouse development, Tbx3 expression begins in the inner cell mass of the blastocyst, and then appears in the extraembryonic mesoderm during gastrulation. During organogenesis, Tbx3 is expressed in the nervous system, skeleton, eye, heart, kidney, lungs, pancreas, and mammary gland.

There are two known isoforms of Tbx3 that result from differential splicing in the second intron, Tbx3 and Tbx3+2a, which includes 20 extra amino acids in the DNA binding domain of the protein. While both have been detected, there is no known unique role for

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one or the other specific isoform in development. A null allele of \textit{Tbx3} has been generated and homozygous mutant mice have defects in a number of structures such as the limbs, mammary glands, and heart. These mutants die by embryonic day (E) 16.5 with greater than 50% dead by E11.5, most likely because of yolk sac defects. A number of different organ-specific effector genes and transcription factors are aberrantly expressed in these mutants.\textsuperscript{7}

In humans, \textit{TBX3} mutations have been linked to unlar-mammary syndrome (UMS, MIM 181450), a disease with variable penetrance characterized by shortened forelimbs, defective apocrine gland and genital development, and heart abnormalities.\textsuperscript{8,9} This phenotype is similar to that seen in \textit{Tbx3} mutant mice, although in humans the phenotype is seen in heterozygotes whereas in mice, only homozygotes have severe defects. The spectrum of affected organs in UMS is characteristic of diseases associated with T-box factors and is indicative of the complex transcriptional networks in which these genes participate during development.\textsuperscript{10} \textit{Tbx3} mutations have also recently been found to impact the pluripotency of embryonic stem cells and the invasiveness of cancer.\textsuperscript{11–15}

For \textit{Tbx3} to play a part in the development of so many different organs, it must interact with a network of genes and proteins specific to each spatial and temporal location of action. While \textit{Tbx3} most likely binds to its target promoters as a monomer, other factors are known to enhance \textit{Tbx3}-mediated transcriptional activation or repression, hinting at a large network of factors that give specificity to \textit{Tbx3} activity.\textsuperscript{16,17} In addition, \textit{Tbx3} has been shown to have both activation and repression domains which may be modulated by other cofactors to ensure the proper function of the protein in each context.\textsuperscript{18} Only by understanding the function of \textit{Tbx3} by a systems approach in a variety of developmental contexts can we hope to unravel the network of genes of which \textit{Tbx3} is a part.

\textbf{Tbx3 in Development}

\textit{Tbx3} in Mammary Gland Development

The initiation and growth of the mammary gland is dependent on fibroblast growth factor (FGF) and WNT signaling and involves reciprocal interactions between the epidermis and the underlying mesenchyme in bilateral ‘milk lines’. Mesenchyme induces the formation of mammary placodes in five specialized areas along each flank of the embryo. The epidermal placode forms a mammary bud which in turn influences the surrounding mesenchyme to form the primary mammary mesenchyme. \textit{Tbx3} is initially expressed in the mesenchyme of the milk line prior to placode formation and then appears in the mammary placodes as one of the earliest markers of mammary placode formation. Expression in the mesenchyme gradually decreases while epithelial expression is maintained.\textsuperscript{1,5,17,20} During late gestation, \textit{Tbx3} is expressed in mammary mesenchyme surrounding the nipple (Figure 1(a)) and in postnatal females it has been detected in virgin, pregnant, lactating and involuting mammary glands.\textsuperscript{21}

UMS in humans is characterized by variable abnormalities of the mammary gland ranging from normal to hypoplastic breasts, with missing or supernumerary nipples. A loss of function mutation of mouse \textit{Tbx3} results in the failure of mammary placode induction in homozygous females and aplasia or a decrease in the extent of branching of the ductal tree in heterozygous females. This effect on the developing mammary gland is independent of the repression of one or the other specific isoform in development.\textsuperscript{7} These mutants die by embryonic day E16.5 and WNT and FGF signaling feed into the \textit{Tbx3} regulatory network. Fgfr2b and Fgfr3/4c are upstream of \textit{Tbx3} expression, and Wnt10b, Lef1, and FGF signaling are all lost in the absence of \textit{Tbx3}.\textsuperscript{19,20} indicating feed-forward and feedback loops of regulation for the maintenance and/or induction of \textit{Tbx3} expression (Figure 2). Similarly, Bmp4 overexpression inhibits \textit{Tbx3} expression in the mammary mesenchyme while, reciprocally, overexpression of \textit{Tbx3} represses Bmp4.\textsuperscript{22} \textit{Tbx3}, in combination with FGF signaling, may be upstream of Nrg1, a growth factor implicated in the initiation of mammary placentation, but the evidence is circumstantial.\textsuperscript{23,24}

The closely related T-box gene, \textit{Tbx2}, is expressed in the mesenchyme but not the epithelium during mammary development and although mutation of \textit{Tbx2} by itself does not result in a mammary gland phenotype, a genetic interaction with \textit{Tbx3} is evident in double heterozygous females by an exacerbation of mammary aplasia.\textsuperscript{19}

\textit{Tbx3} in Limb Development

In vertebrates, limbs develop as a set of lateral bulges from the lateral plate mesoderm on either side of the body axis. The initial events in limb development involve proliferation of the lateral plate mesoderm and induction of the apical ectodermal ridge (AER).\textsuperscript{25–27} Three signaling centers, the AER, the zone of polarizing activity (ZPA) and the nonridge ectoderm, are necessary for growth and patterning of limb buds, processes which involve complex signaling through the FGF and Sonic hedgehog (SHH) pathways.\textsuperscript{28} All four
FIGURE 1 | Expression of Tbx3 (blue) in developing organ systems at different stages. (a) In mammary gland, Tbx3 is first expressed at E10.5 in the mesenchymal milk line and then appears as one of the earliest markers of the epithelial thickenings known as the mammary placodes. It continues to be expressed in the epithelium as the placode expands into the mammary bud and eventually forms the branching ductal system. Near term (E18.5), mesenchyme surrounding the nipple expresses Tbx3. (b) Tbx3 is first expressed in the posterior margin of the early limb buds and then in the posterior and anterior margins of both fore and hind limbs by E10.5. It is also expressed in the AER, continuously at first and then limited to the tips of the digits by E12.5. (c) Tbx3 is expressed in the AVC, SAN, OFT and atrioventricular bundle (AVB) starting around E10.5. It fully delineates the cardiac conduction system at E14.5 with expression in the SAN, AVN, AVB, and the bundle branches (BB).

members of the Tbx2 subfamily are expressed during limb development. In mice, Tbx3 expression is first detected at the posterior margin of the early limb buds, and shortly thereafter in the anterior and posterior proximal mesenchyme and AER. As the limb bud elongates, Tbx3 anterior and posterior expression domains are expanded in the mesenchyme. By E13.5, expression in the AER is limited to the tips of the digits25-27 (Figure 1(b)). A similar pattern is observed in the chick.28-31 In UMS, posterior structures of the fore limb, for example, the ulna and the fifth digit are missing.8 Mice homozygous for the Tbx3 null allele similarly exhibit missing or abnormal posterior fore limb
elements, but unlike UMS also show severe hind limb abnormalities.7

Little is known about the direct regulation of Tbx3 in limb development. Studies in the chick indicate that Tbx3 expression in the posterior of the limb buds is controlled via different mechanisms than in the anterior. The posterior domain of Tbx3 expression depends on the ZPA signaling cascade and is regulated positively by Shh, but the anterior expression domain is negatively regulated by Shh and is dependent on continuous signaling by anteriorly produced BMPs, suggesting a potential role for Tbx3 in the antero-posterior patterning of the limb.31 A recent study places retinoic acid (RA) signaling upstream of Tbx3 in the limbs (RD Ballim, C Mendelsohn, VE Papaioannou, S Prince, personal communication). In mice, Shh and Hand2 appear to be downstream targets of Tbx3.7 Studies in chick have implicated Tbx3 in positioning the limb along the main body axis through a genetic interplay between Hand2 and Gli3, but the interrelationship of these genes is not clear.32 Inactivation of Dicer in mice

FIGURE 2 | Diagram of known regulatory pathways and downstream targets of Tbx3 in the development of heart, mammary gland and limbs, as well as in embryonic and iPS stem cells. The variety of factors involved illustrates the context-dependent nature of Tbx3 interactions.
results in a posterior shift and a delayed formation of hind limb bud which is accompanied by altered transcription of Tbx3, Hand2 and Gli3. This study showed that microRNA is also capable of inhibiting Tbx3 and Hand2 expression in vitro. Hence, Tbx3 and Hand2 might be downstream of Dicer-mediated regulation in limb bud positioning.53 (Figure 2).

Tbx2 has a similar spatiotemporal expression pattern in limb buds in both chick and mice27,29–31 and is downregulated in Tbx3 mutants.7 Experiments in the chick have shown that Tbx3 and Tbx2 together specify the identity of posterior digits, acting through regulation of interdigital BMP signaling,14 possibly indicating a genetic interaction.

**Tbx3 in Heart Development**

The transformation from linear heart tube to the four-chambered heart is accomplished by the differential cell growth and distinct gene programs adopted by different regions in the heart. Starting at E9.5, the working myocardium cells undergo rapid and sustained proliferation to form the muscular chambers of the heart. The intervening regions of non-chamber myocardium, meanwhile, are held relatively mitotically inactive to form the constrictions between the chambers that will eventually become components of the cardiac conduction system (CCS).

Tbx3 expression is first detected in the heart at E8.5 and as the heart undergoes looping Tbx3 expression delineates the developing nodal conduction system with expression in the sinoatrial node (SAN) and atrioventricular node (AVN), as well as the endocardial cushions in the atrioventricular canal (AVC) and the mesenchyme of the outflow tract (OFT) (Figure 1c(i)). This expression pattern is almost identical to that of Tbx2 although no genetic interaction has been demonstrated in this tissue. Tbx3 is thought to have two distinct roles in the developing CCS: first, the modulation of cell division resulting in constrictions between chambers, and secondly, the repression of a chamber-specific gene program and concomitant promotion of a conduction system-specific gene program. Despite the assumption that Tbx3 mutant embryos die at midgestation due to yolk sac deficiencies, their hearts have altered morphology including double outlet right ventricle, incomplete ventricular septation, and delayed aortic arch formation.35 These malformations are a result of increased cell division in the AVC and OFT leading to a lack of constriction.36 Mutant hearts also have ectopic expression of chamber myocardium genes, such as Cx40, Cx43, and Nppa, in the non-chamber AVC, a phenotype resembling that of Tbx2 mutants. Conversely, CCS-specific genes Hcn4 and Ldb1 are upregulated in regions where Tbx3 ectopic expression is induced, and functional conduction tissue develops37 (Figure 2).

On a protein level, it appears that Tbx3 regulates its targets by cooperatively binding their promoters along with other transcription factors. For example, Tbx3 has been shown to bind cooperatively with Msx1 and Msx2 in the repression of Cx43.16 Similarly, Tbx2 has been shown to bind to Nkx2.5 and repress Nppa, a known Tbx3 target. However, in the absence of Tbx2, Tbx3 binds to Nkx2.5 and activates Nppa14 (Figure 2). This suggests a regulatory mechanism whereby binding competition with a network of transcription factors determines which gene program will be expressed in a given tissue.

Tbx3 mutant heart abnormalities result from increased cell division in the regions of Tbx3 expression implicating Tbx3 in the regulation of cell dynam- ics in the process of heart looping and growth. Conversely, despite its role in the regulation of the gene expression profile of the CCS, Tbx3 mutant hearts have normal conduction velocity and several of the conductive structures are present. This discrepancy is likely due to the functional overlap of Tbx3 with Tbx2, which has been shown to bind to and regulate many of the same targets. Nonetheless, some patients with UMS show conduction defects in line with abnormal development of conduction structures.7 These defects are similar to those in mice mutant for Tbx2, highlighting the potential functional overlap with Tbx3 in the development of the CCS.39

**Tbx3 in STEM CELL BIOLOGY**

In addition to its key roles in development, Tbx3 also plays a role in both the establishment and maintenance of pluripotency in embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. ES cells are derived from the inner cell mass (ICM) of preimplantation blastocysts and rely on the LIF/STAT3 pathway to maintain pluripotency. In the embryo, Tbx3 is first expressed in the ICM4 and this expression is recapitulated in ES cells. Tbx3 expression is highest when ES cells are undifferentiated and decreases as cells differentiate into embryoid bodies, suggesting its importance in the maintenance of pluripotency.13

In ES cells, Oct4 and Nanog, two recognized markers of pluripotency, act as repressors of differentiation toward a trophectoderm and endodermal fate, respectively. Similarly, Tbx3 is able to block differentiation into mesoderm, ectoderm, trophectoderm, and neural crest cell fates.11,13 ES cells treated with shRNA against Tbx3 downregulate both Oct4 and Nanog, and show differentiated morphology and reduced
alkaline phosphatase activity. To function as a mediator of pluripotency, Tbx3 is able to act with Klf4 to regulate the expression of Nanog specifically, lying at the center of a LIF-independent pluripotency pathway in ES cells. In addition to blocking differentiation, Tbx3 also appears to play a role in the differentiation of ES cells into extraembryonic endoderm (ExEn) as overexpression of Tbx3 in ES cells induces differentiation into cells with ExEn morphology as well as expression of ExEn markers such as Gata6. This dual functionality suggests that Tbx3 takes part in a complex regulatory network where it is able to function both as a repressor of specific cell fates and an activator of others. In this way, ES cells are poised to differentiate into a given cell type quickly when the proper signals are received: the relief of one repression module allows the activation of another. The complexity of Tbx3 in the pluripotency network is evident as the promoter of Tbx3 itself is bound by a number of transcription factors at the core of the genetic regulation circuit of pluripotency (Figure 2). Mechanistically, Tbx3 is able to regulate transcription at the level of DNA, but also on an epigenetic level: Tbx3 binding to the Gata6 promoter is necessary to activate transcription but Tbx3 is also able to mediate the histone methylation of H3K27me3 at the Gata6 promoter.

In addition to the maintenance of pluripotency, Tbx3 may also play a role in the establishment of pluripotency in iPSCs. Fibroblasts with induced expression of Tbx3 in combination with the reprogramming factors Sox2, Oct4, and Klf4 express pluripotency markers more rapidly than fibroblasts without. Moreover, iPSCs cells with induced Tbx3 expression contributed to enhanced germ line contribution and transmission.

Tbx3 IN CANCER

Tbx3 is amplified and/or overexpressed in many tumors (Table 1). Accumulating evidence suggests that Tbx3 contributes to tumorigenesis through interaction with components of several major oncogenic pathways (Figure 3), some with which Tbx3 is known to interact in other contexts. Activation of the canonical Wnt-β-catenin pathway has been linked to many types of cancer. β-Catenin plays dual roles depending on intracellular localization: in the nucleus it acts as the main effector of WNT signaling and at the plasma membrane as a component of adherens junctions where it links E-cadherin with the actin cytoskeleton. Tbx3 is a downstream target of the Wnt-β-catenin pathway in liver tumorigenesis, and recent evidence suggests that there is a feedback loop by which Tbx3 can upregulate β-catenin. Thus, Tbx3 could be a critical mediator of cellular responses to proliferative and anti-apoptotic signals delivered by β-catenin. Interestingly, Tbx3 represses E-cadherin, which has been implicated in metastasis of invasive epithelial tumors. Together these findings suggest that Tbx3 can enhance tumor invasiveness through both E-cadherin repression and β-catenin upregulation. Additionally, phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment leads to downregulation of E-cadherin, and as TPA activates TBX3 in a PKC-dependent manner, it is possible that upregulation of TBX3 is mediating this process.

As in normal mammary gland development, FGF signaling is upstream of TBX3 expression in breast cancer. Moreover, estrogen can upregulate TBX3 levels in breast cancer via paracrine FGF9-FGFR3 signaling and the upregulation of TBX3 expands the pool of functional estrogen receptor (ER)-negative cancer stem-like cells. This implies that resistance to anti-estrogen therapy which is common in breast cancer might be accompanied by an increase in FGF-TBX3 signaling and a consequent increase in the proportion of cancer stem-like cells. Thus, targeting of the FGF-TBX3 pathway could be a useful strategy for refractory breast cancers. Moreover, TBX3 can affect the equilibrium of cell type differentiation within breast epithelial cancers, which is context-dependent for a given cancer cell population. Together these studies suggest that TBX3 could play important roles in cell plasticity within breast cancer.

Upregulation of Tbx3 suppresses the expression of ARF (p19ARF in mouse and p14ARF in humans) and possibly p16INK4a and promotes the bypass of senescence through inactivation of p53 via ARF-MDM2-p53 tumor suppressor pathway. Tbx3 can also directly repress the p21 promoter and bypass senescence independently of p53. The knockdown of Tbx3 in both melanoma and breast cancer cell lines leads to reduction in anchorage-independent growth, migration and tumor formation, and a decrease in pro-senescence factors that results in increased proliferation. It was previously suggested that Tbx3 and its splice variant Tbx3+2a, are functionally distinct in inhibition of senescence. However, a subsequent study convincingly demonstrated that both isoforms function as anti-senescence factors, bind the same T-half-site and possibly anti-senescence factors, bind the same T-half-site and possibly
TABLE 1 | Incidence of Tbx3 Expression in Human Cancers and Corresponding Normal Tissue in the Mouse

<table>
<thead>
<tr>
<th>Cancer</th>
<th>No. (%) of Specimens with Tbx3 Expression</th>
<th>Method of Detection</th>
<th>Corresponding Normal Expression of Tbx3</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>48/50 (96)</td>
<td>WB and real time PCR</td>
<td>Mammary epithelium and mesenchyme of developing gland</td>
<td>42,44,51</td>
</tr>
<tr>
<td>Melanoma</td>
<td>7/12 (58)</td>
<td>WB</td>
<td>Melanocytes1</td>
<td>47</td>
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<tr>
<td>Pancreatic</td>
<td>48</td>
<td>Microarray</td>
<td>Developing pancreas</td>
<td>53,55,59</td>
</tr>
<tr>
<td>Ovarian</td>
<td>21/29 (70)</td>
<td>MALDI-Tof-MS</td>
<td>Developing pancreas</td>
<td>44</td>
</tr>
<tr>
<td>Prostate</td>
<td>ND</td>
<td>GWAS</td>
<td>Developing pancreas</td>
<td>42,43,49</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>ND</td>
<td>Microarray</td>
<td>Developing CNS</td>
<td>54</td>
</tr>
<tr>
<td>Pheochromocytoma</td>
<td>ND</td>
<td>Microarray</td>
<td>Developing CNS</td>
<td>42,48</td>
</tr>
</tbody>
</table>

GWAS, genome-wide association study; ND, not determined; MALDI-Tof-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; RT-PCR, reverse transcriptase–polymerase chain reaction; WB, western blot.

Cancers listed are those in which Tbx3 has been shown to be amplified and/or overexpressed.

1 Melanoma cell lines in vitro.
2 Also present in human melanocyte cell lines.

FIGURE 3 | The Tbx3 interactome in cancer. Known and hypothetical molecular interactions between Tbx3 and components of several signaling pathways important in oncogenesis are drawn from a variety of contexts.

putative Tbx3 downstream targets in breast cancer.66 Although chromatin immunoprecipitation analysis confirmed direct binding of Tbx3 to both of these targets, the functional significance of these findings is not known. Interestingly, GATA3 was shown to inhibit breast cancer metastasis by directly upregulating E-cadherin levels.67 It is tempting to speculate that Tbx3 could be repressing GATA3 or alternatively affecting E-cadherin levels by binding to both GATA3 and E-cadherin. Gli3 belongs to the hedgehog (Hh) signaling network and is required for normal mammary bud formation.68 As deregulation of Hh...
pathway is implicated in a wide variety of aggressive and metastatic cancer, the predicted Tbx3–Gli3 interaction warrants further investigation.

CONCLUSION
The Tbx3 transcriptional network is highly context dependent. This flexibility allows the protein to assume different functions that are specialized for the time and place of expression. Nonetheless, there are common themes that run through the network that hint at more general functions for the gene. In the heart and ES cells, Tbx3 blocks the differentiation of multipotent tissues. This inhibition of differentiation may play a role in cancers when Tbx3 is overexpressed or amplified: induction of an undifferentiated ‘stem-like’ cancer cell by Tbx3 may initiate the process of tumor formation and cell migration. This repressive function is evident in in vitro assays where a transcriptional repression module has been noted.69 Conversely, Tbx3 can induce differentiation in different contexts. In ES cells, for example, Tbx3 promotes differentiation into ExEn. In the mammary gland as well, Tbx3 induces differentiation of the mammary placodes. Indeed, by binding to tissue-specific transcription factors, Tbx3 may be able to either repress or activate the differentiation of multipotent progenitors in a context-dependent manner.

Tbx3 also appears to play a role in cell proliferation in a number of different contexts: in the heart, Tbx3 depletion leads to an excess of cell proliferation that is largely recapitulated in human UMS. This role is highlighted in cancers where TBX3 is overexpressed or amplified as it results in the bypass of senescence through inactivation of the p53 pathway, while the knockdown of Tbx3 leads to an increase in proliferation.

Finally, Tbx3 appears to play a role as a mediator of cellular signaling by modulating a number of signaling pathways. Tbx3 can control WNT signaling in the mammary gland and limb buds, as well as in various cancer models. FGF and SHH signaling are also modulated by Tbx3 in various contexts. As with cell proliferation, Tbx3 may be able to regulate these pathways generally, but rely on specific signals to impart specificity to this function.

In order for it to assume such distinct functions, Tbx3 interacts with other factors to give a regional and temporal specificity to its action. Given the evidence of Tbx3 functioning in protein complexes with transcription factors of myriad different families and as a competitor for binding to transcriptional targets, it is reasonable to conclude that Tbx3 is able to mediate a specific set of activities, but that available cofactors determine how it will act in specific contexts. The necessity of these cofactors in determining what function Tbx3 will have makes it an important target for studying with a systems-based approach.

REFERENCES


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FURTHER READING