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Delayed differentiation in embryonic stem cells and mesodermal progenitors in the absence of CtBP2

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ABSTRACT

Mammalian embryonic stem cells (ESCs) are characterized by an ability to self-renew and give rise to each of the three germ layers. ESCs are a pluripotential source of numerous primitive progenitors and committed lineages and can make stoichiometric decisions leading to either asymmetric or symmetric cell division. Several genes have been identified as essential for maintenance of self-renewal, but few non-lineage specific genes have been identified as essential for differentiation. We selected the chromatin factor *Ctbp2* from microarray data for its enriched expression in stem cells, in comparison to committed progenitors. RNA interference (RNAi) was used to knockdown gene expression in mouse ESCs and the potential for transduced cells to self-renew and differentiate was assessed in ESC and mesodermal assays. Here, we demonstrate an important role for *Ctbp2* in stem cell maintenance and regulation of differentiation using an in vitro system. The knockdown of *Ctbp2* increases the prevalence of ESCs in culture, delays differentiation induced by LIF withdrawal, and introduces developmental changes in mesodermal differentiation. A model is presented for the importance of *Ctbp2* in maintaining a balance in decisions to self-renewal and differentiate.

1. Introduction

C-terminal binding protein (CtBP) was identified because of its interaction with the C-terminus of the adenovirus E1A oncoprotein (Boyd et al., 1993; Schaeper et al., 1995). In adenovirus, CtBP represses E1A transcriptional activation and oncogenesis (Sollerbrant et al., 1996). Detailed reviews of CtBP's isolation, evolutionary conservation across eukaryotes, and biochemical activity have been published by Turner and Crossley (2001) and Chinnadurai (2002). Briefly, CtBP proteins are highly conserved across *Drosophila*, zebrafish, mice, and humans. *Drosophila* CtBP (dCtBP) interacts physically and genetically with the transcriptional repressors *kruppel*, *knirps*, and *hairy* in early development (Hildebrand and Soriano, 2002). In *Drosophila*, CtBP2 also interacts with *snail*, which is required for proper mesodermal development and cell fate decisions (Hemavathy et al., 2004).

The two CtBP proteins in mice – CtBP1 and CtBP2 – differ in expression, cellular localization, and regulatory role. *Ctbp1* is expressed throughout embryogenesis and into adulthood. *Ctbp2* is expressed in the forming placenta and is restricted to embryogenesis (Furusawa et al., 1999). CtBP1 is cytoplasmic and CtBP2 is found almost exclusively in the nucleus (Zhao et al., 2006). Both mammalian CtBPs associate with Polycomb proteins and complex with transcription factors to repress genes involved in mouse development. They have roles that

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somewhat overlap but they have differing degrees of importance. *Ctbp1* nulls are born in expected Mendelian proportions. *Ctbp2* nulls are embryonic lethal with developmental abnormalities appearing by E10.5 (Hildebrand and Soriano, 2002). The primary causes of lethality are defects in placental and yolk sac development. There is also an observed decrease in *Brachyury* expression. CtBP2 appears to be the more critical of the two CtBPs in embryonic development, as *Ctbp1* nulls with a heterozygous *Ctbp2* background are not viable and die around E15.5–18.5 (Hildebrand and Soriano, 2002).

CtBP2 interacts physically with key factors in the stem cell regulatory network. SMAD6 recruitment of CtBP2 to repress BMP-associated transcription has been demonstrated in vitro and in vivo (Lin et al., 2003; Hassel et al., 2004). CtBP2 can also bind SOX6 and repress transcription of *Fgf-3* (Murakami et al., 2001). Other stem cell-related transcription factors that associate with CtBP2 to co-repress transcription include the AML/EVI1 oncoprotein, the Gata1-binding factor known as Friend of Gata (FOG), the mesodermal and ectodermal-specific transcription factor γ-EF1, and Krupple-like factor 3 (KLF3) (Turner and Crossley, 1998; Fox et al., 1999; Furusawa et al., 1999; Holmes et al., 1999; Turner et al., 2003). Although these protein–protein interactions have been documented biochemically, there are few studies that describe the functional importance of CtBP2 in mammalian embryonic development. Based on the in vivo description of embryonic lethality in *Ctbp2* nulls and heterozygotes and based on decreased expression of *Brachyury*, we predict that *Ctbp2* is important in early embryonic differentiation and proper mesodermal emergence. In this study, we use ESC and blast assays, as in vitro proxies for the blastocyst and mesoderm, respectively, with the objective of providing an in vitro parallel of what happens developmentally prior to E10.5 in a *Ctbp2* null.

2. Results

2.1. *Lentiviral-mediated RNA interference of CTBP2 is efficient*

Lentiviral RNA interference vectors were designed to knockdown expression of *Ctbp2* in mouse ESCs (mESCs). A lentiviral RNAi vector against *Oct4*, provided by Dr. Natalia Ivanova at Princeton University, was used to transduce mESCs in order to generate a self-renewal control and lentiviral vectors against *Smarca4* were used to generate a cell-cycle control. RNAi was successful with all vectors, however, the vectors containing shRNA sequences designed against the more 3′ region of the open reading frame (ORF) were most efficient. The *Ctbp2*-A vector, which targets the more 5′ region of the *Ctbp2* ORF, decreased gene expression by a broad range of 28–54% (Fig. 1A). The *Ctbp2*-B vector, which targets the more 3′ region of the ORF, decreased gene expression by a nar-

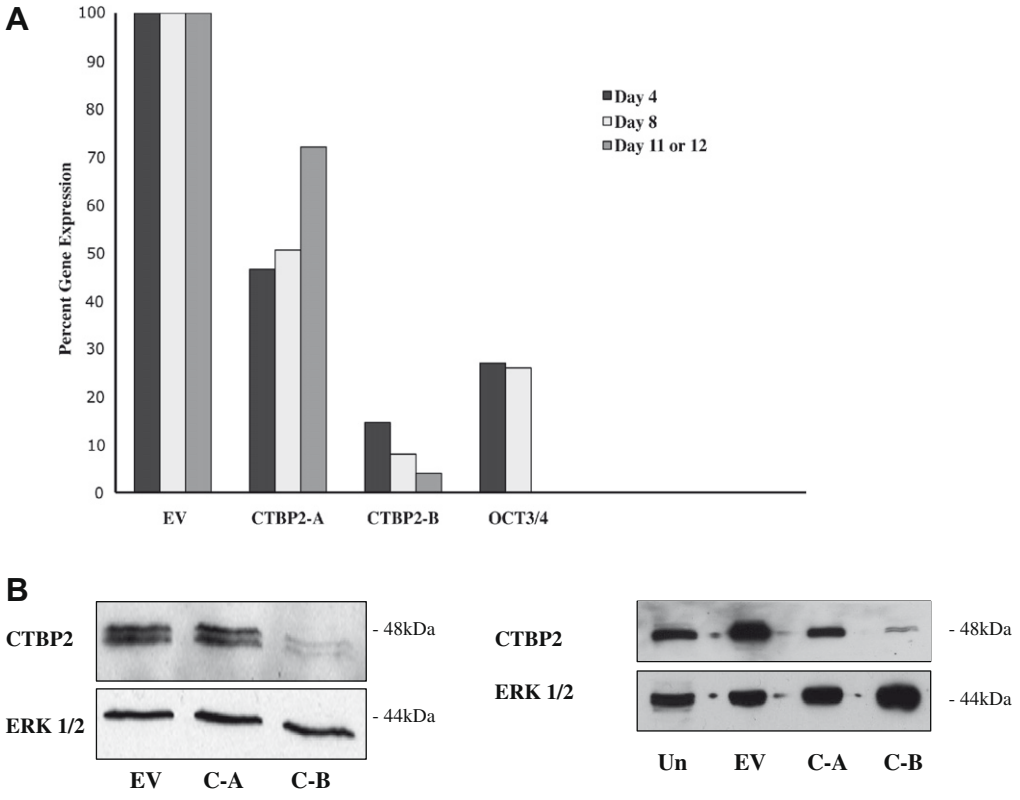

Fig. 1 – (A) Real-time PCR (RT-PCR) confirmation of *Ctbp2* RNAi, as compared to empty vector (EV) and *Oct4* RNAi positive control. The *Ctbp2*-A RNAi construct targets a more 5′ region of the open reading frame (ORF) of *Ctbp2* and the *Ctbp2*-B RNAi construct targets a more 3′ region of the ORF of *Ctbp2*. Gene expression is the average per timepoint from two independent experiments. (B) Western blots to confirm knockdown of CtBP2 protein in uninfected (Un), empty vector (EV), *Ctbp2*-A RNAi (C-A) and *Ctbp2*-B RNAi (C-B) cultures. Antibodies used against whole cell extracts from cultures at Day 12 (left) and Day 15 (right) post-transduction (p.t.). Each blot represents an independent experiment. ERK-1/2 is used as input control.

rower and more specific range of 85–98% (Fig. 1A). The *Oct4* RNAi control decreased gene expression consistently by 75%, which is sufficient to recapitulate the in vitro and in vivo phenotypes. The efficiency of CtBP2 knockdown was confirmed with Western blots of whole cell extracts from cells sorted into media at Days 12 and 15 post-transfection. Blots show that there is no observable change in CtBP2 with *Ctbp2*-A RNAi, however, there is a clear knockdown of CtBP2 observed with *Ctbp2*-B RNAi (Fig. 1B). The Western blot data is consistent with the quantification of each vector's ability to knockdown *Ctbp2*, as measured by RT-PCR.

2.2. *Self-renewing ESCs are more prevalent in the absence of CtBP2*

After confirming the efficiency of CtBP2 knockdown, transduced ESCs were placed in a competition assay to characterize self-renewal ability. The premise of the assay, as described in more detail in Section 4, is to monitor the ratio of GFP−/GFP+ ESCs over time. The competition assay was read using FACS at 48-h intervals that overlapped with culture passage. The raw percentage change in GFP−/GFP+ is depicted in Fig. 2A and was normalized against the mock infected and empty vector cultures to adjust for an expected ~10% decrease in cells. Our findings are based on cumulative changes in GFP−/GFP+ from Day 2 through Day 14 of competition and indicate that a knockdown of CtBP2 causes an increase in the ratio of GFP−/GFP+ cells (Fig. 2A). The extent of the phenotype is related to the degree of RNAi associated with the *Ctbp2*-A and *Ctbp2*-B vectors. In *Ctbp2*-A RNAi cultures, 45–80% of *Ctbp2* is expressed but without change to protein levels. The absence of change in protein levels is associated with an insignificant increase in the GFP ratio ($p > 0.05$) (Fig. 2A). In contrast, *Ctbp2*-B RNAi reduces *Ctbp2* expression to less than 15% and there is an observable loss of protein. This loss of protein is associated with a significant ($p < 0.05$) 20% increase in the GFP ratio (Fig. 2A).

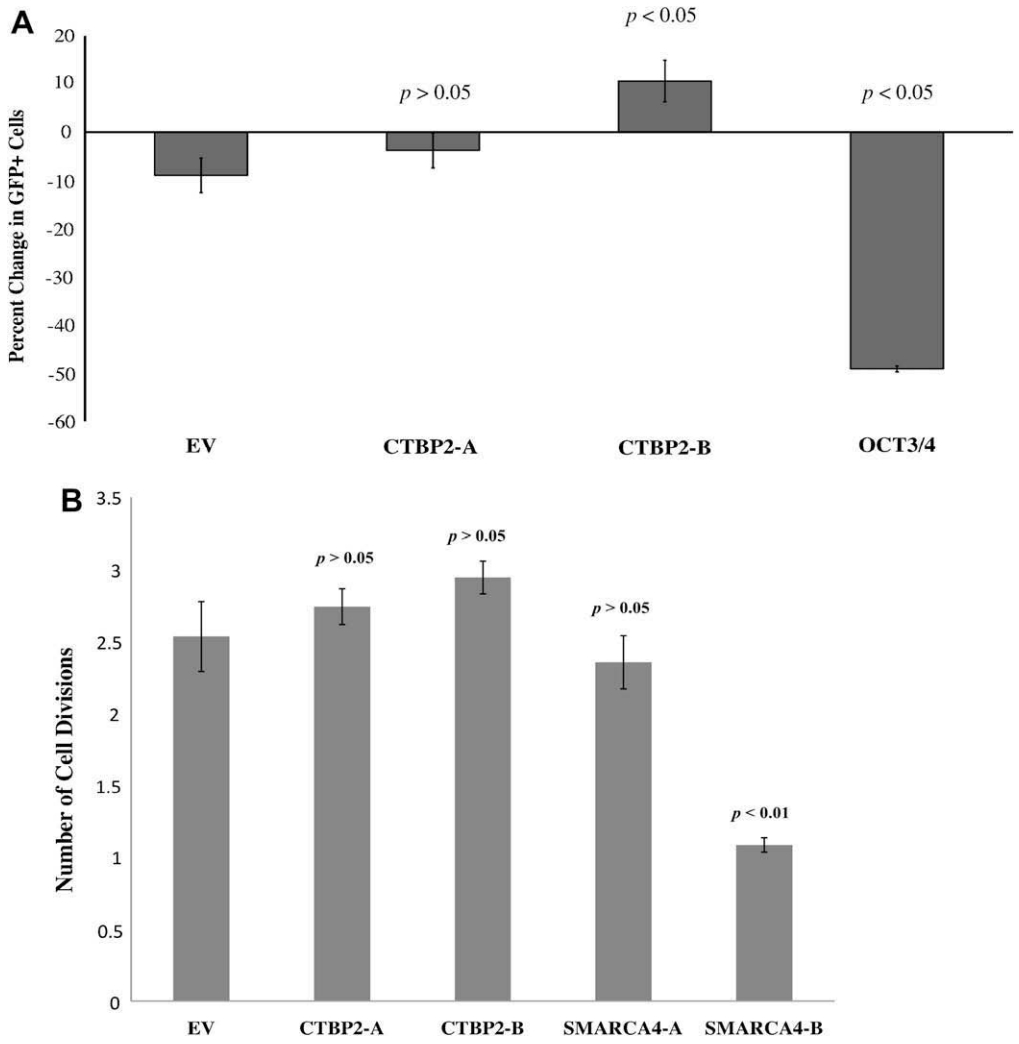

Fig. 2 – ESC self-renewal and maintenance. (A) Percent change in RNAi GFP+ ESCs in competition with GFP− ESCs as measured by FACS. Percent change is cumulative and measured from Day 2 through Day 14 of a timecourse with empty vector (EV), *Ctbp2*-A RNAi, *Ctbp2*-B RNAi, and *Oct4* RNAi cultures. Data presented is the average from four independent experiments. (B) Proliferation assay for a 36-h culture period with empty vector (EV), *Ctbp2*-A RNAi, *Ctbp2*-B RNAi, *Smarca4*-A RNAi and *Smarca4*-B RNAi cultures. Data presented is the average from four independent experiments.

Since *Ctbp2*-B RNAi cells experience an apparent gain in self-renewal ability and increased prevalence in culture, then the assumption is that either the progression through the cell cycle quickens or the balance between asymmetric and symmetric self-renewal is altered. To explore the former possibility, we performed PKH26 dye and Annexin V staining assays using *Smarca4* RNAi cultures as a positive control. We did not observe significant cell cycle-related changes in *Ctbp2* RNAi cultures although it was apparent that independent cultures of *Ctbp2*-B RNAi ESCs produce greater numbers of ESCs over time. In a proliferation assay with *Ctbp2*-B RNAi, we did not observe a significant ($p > 0.05$) increase in cell division (Fig. 2B) or change in STAT3 and AKT signaling (Supplementary materials, Fig. S1). In the absence of data in support of increased cell division in *Ctbp2* RNAi ESCs, we turned to assays that would explore the possibility of a shift in self-renewal and differentiation.

We used three methods to characterize self-renewal and differentiation – AP assay, colony measurements, and LIF withdrawal. An increase in the self-renewal ability of ESCs should affect colony morphology and maintenance. Changes in colony morphology can be visualized in an alkaline phosphatase (AP). In the AP assay for *Ctbp2* RNAi, we analyzed the morphology of *Ctbp2*-B RNAi ESCs in LIF+ (self-renewal) and LIF− (differentiation) conditions. In the presence of LIF, we observed *Ctbp2*-B RNAi colonies that were larger in comparison to the controls and in comparison to *Ctbp2*-A RNAi (Fig. 3A). We used IPLab software to trace colony boundaries and to select the pixels at colony boundaries to generate measurements of perimeter and area. Our findings of larger colony area and perimeter for *Ctbp2*-B RNAi cultures confirm the results of the AP assay (Fig. 3B). In the AP assay in the absence of LIF, differentiation ensues as expected in all cultures except for *Ctbp2*-B RNAi. In the *Ctbp2*-B RNAi cultures, large round colonies are still visible in contrast to mock, empty vector (EV), *Ctbp2*-A RNAi and *Oct4* RNAi cultures (Fig. 3A). This difference in colony morphology of *Ctbp2*-B RNAi is consistent with the demonstrated difference in degree of gene expression knockdown and significance of competition ratios. The morphological observation in the LIF− panel of the AP assay is supported by analysis of *Oct4* levels during a LIF withdrawal timecourse. In the AP assay when LIF is removed, *Ctbp2*-B RNAi cultures do not differentiate as quickly as control cultures (Fig. 3A, compare LIF to No LIF). After 7 days without LIF, the EV cultures can be categorized as "differentiated" and the *Ctbp2*-B RNAi cultures resemble the "semi-differentiated" cultures pictured in Ivanova et al. (2006). The morphological delay in differentiation is confirmed by a corresponding delay in decrease of *Oct4* expression after LIF withdrawal. In Fig. 3C, we show that *Oct4* levels decrease sharply and as expected in empty vector cultures by Day 4 of LIF withdrawal, whereas *Oct4* levels in *Ctbp2*-B RNAi cultures do not decrease sharply and are not at comparably low levels until Day 6 of differentiation.

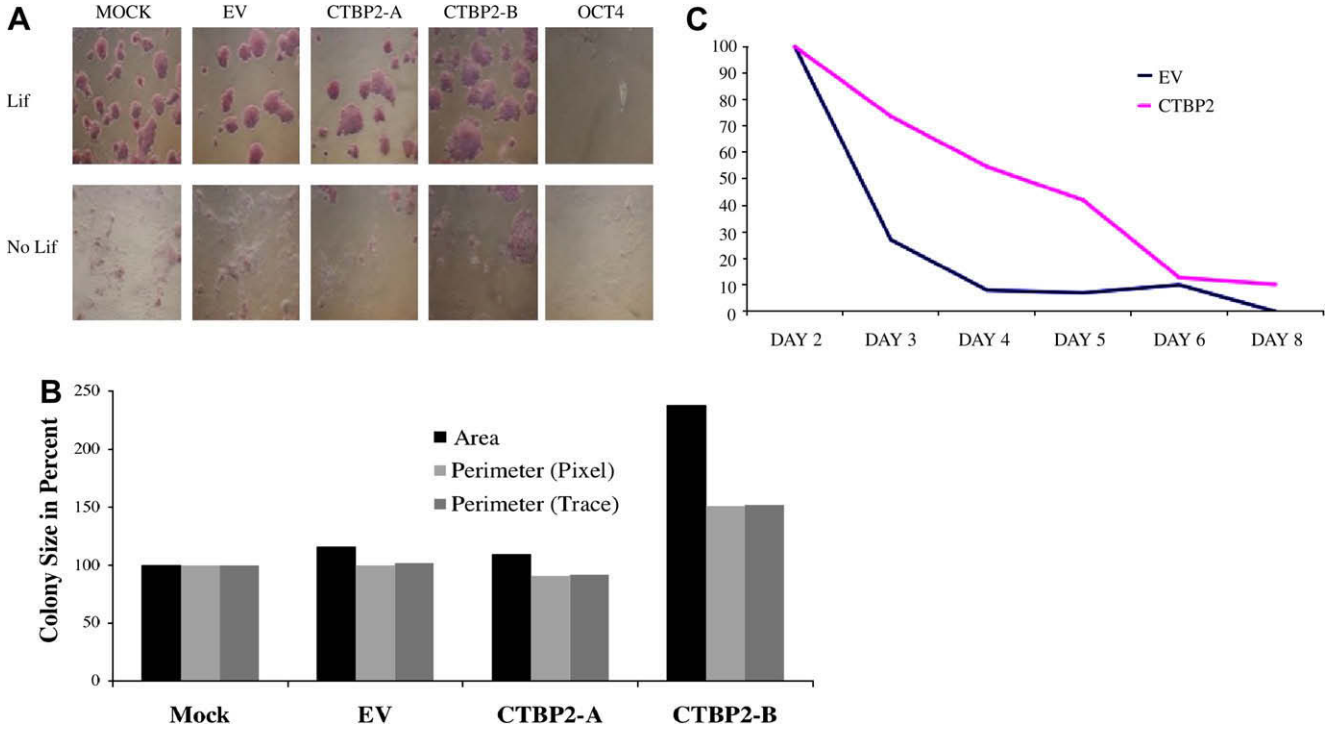

Fig. 3 – (A) Alkaline phosphatase (AP) assay of ESCs in self-renewal (LIF+, top) and differentiation (LIF−, bottom) conditions. Cells were sorted for GFP expression at Day 2 post-transduction (p.t.), plated in their respective conditions at Day 4 p.t., and assayed at Day 9 p.t. (B) Average size of colonies from *Ctbp2*-A RNAi ($n = 15$) and *Ctbp2*-B RNAi ($n = 11$) cultures in comparison to mock infected (Mock, $n = 20$) and empty vector (EV, $n = 14$) cultures, as measured using IPLab software. (C) Expression timecourse of *Oct4* in empty vector (EV) and *Ctbp2*-B RNAi cultures, measured by RT-PCR, after differentiation induced by LIF withdrawal at Day 0.

2.3. Blast colony formation is delayed in Ctbp2 RNAi cultures

Our findings in ESC self-renewal assays demonstrate that a decrease in CtBP2 affects stem cell maintenance by increasing the prevalence of ESCs in culture and shifting cell fate decisions away from differentiation. We wanted to further characterize the impact of *Ctbp2* RNAi on differentiation and chose mesodermal differentiation assays given reported in vivo changes in yolk sac development and *Brachyury* expression in the null (Hildebrand and Soriano, 2002).

The transferability and efficiency of RNAi in the in vitro mesodermal differentiation assays was verified using a proof-of principle experiment targeting *Aml/Runx1*. In vivo, *Aml/Runx1* is required for the development of definitive, but not primitive, hematopoietic lineages from the hemangioblast (Okuda et al., 1996; Wang et al., 1996). Lentiviral-mediated RNAi against *Aml/Runx1* recapitulates the in vivo phenotype and results in normal primitive and blocked definitive hematopoiesis (Supplementary materials, Fig. S2A). The RNAi vector is stable in the hemangioblast system and expression from the RNAi cassette is maintained throughout mesodermal induction and differentiation (Supplementary materials, Fig. S2B). We used the *Ctbp2*-B RNAi vector for the experiments in the hemangioblast system because this vector yielded the highest level of knockdown and the most significant results in the ESC assays.

Formation of mesodermal progenitors, known as hemangioblasts (in vivo) or blast colonies (in vitro) is delayed in *Ctbp2*-B RNAi cultures (Fig. 4A). Upon differentiation and during the blast formation window of Days 2.5–3.5, C-KIT expression should decrease and FLK-1, a marker for blast colonies, should increase. At Day 3.5 when blast colony formation is expected to peak, C-KIT expression should rebound because *c-kit* is necessary for hematopoietic development. CD31 expression should decrease through Day 3.5. The pattern of marker expression at Day 3.5 is very specific and critical because it predicts the emergence of the in vitro hemangioblast (Lacaud et al., 2004). Blast colonies can only form properly if self-renewal has been sufficiently suppressed. A temporal comparison of the empty vector cultures (EV RNAi) and *Ctbp2*-B RNAi shows a clear half-day delay in kinetics, even though EV RNAi and *Ctbp2*-B RNAi cultures are similar at Day 0 (Fig. 4B). This confirms that neither has entered differentiation prematurely. A snapshot of FACS kinetics at Day 3.5 shows that EV RNAi has the expected expression pattern of FLK-1 and C-KIT expression and that CD31 expression has decreased as expected (Fig. 4A). In contrast, the C-KIT expression in *Ctbp2*-B RNAi has not rebounded and the expression of CD31 is lagging in its descent. Quadrant statistics on biological replicates of the FACS timecourse confirm that the differences in FLK-1 and C-KIT expression between EV RNAi and *Ctbp2*-B RNAi at Days 2.5, 3, and 3.5 are significant ($p < 0.05$) with the largest difference at Day 3.5 (Fig. 4B). CD31 expression is also significantly different between the two populations at Days 3 and 3.5 (Fig. 4B, $p < 0.05$).

The FACS kinetics for expression of FLK-1, C-KIT and CD31 define the window of blast colony formation, but the colony-forming assays confirm blast colony emergence during this window. The morphology of the blast colonies and blast precursors (secondary EB and transitional) do not differ between normal and RNAi cultures (Supplementary materials, Fig. S3). We found that there are significantly more ($p < 0.05$) mesodermal progenitor colonies in *Ctbp2*-B RNAi cultures at Day 3.5 with the largest difference in the Flk+ sorted population (Fig. 5A). It is important to note that the Flk+ population gives rise to the blast colony; however, when we characterized the colonies according to type we found that although *Ctbp2*-B RNAi has a larger total number of colonies, it has a much lower number of mature blast colonies. The bulk of the total colony count consists of immature transitional colonies and undifferentiated secondary EBs (Fig. 5B).

If we look at the distribution of the three colony types (blast, transitional and secondary EB) over time, we can characterize a difference in the pattern and distribution of blast colony emergence with a significant difference ($p < 0.05$) in the number of blast colonies at Day 3.5 in *Ctbp2*-B RNAi (Fig. 6). The EV RNAi cultures peak in blast formation at Day 3.5, as expected, but the *Ctbp2*-B RNAi cultures do not experience a peak in blast colony formation until Day 4. At the time of EV RNAi peak (D3.5), blast colonies represent roughly 60% of total colonies, but at the time of *Ctbp2*-B RNAi peak (D4), blast colonies are in the minority at approximately 30% of total colonies. The kinetics of *Brachyury* expression, conducted in quadruplicate, correlate well with the FACS profiles. *Brachyury* expression in EV RNAi peaks at Day 3 of mesodermal induction and precedes the point of hemangioblast formation at Day 3.5, as expected (Fig. 7). *Brachyury* expression in *Ctbp2*-B RNAi is down regulated and expression does not reach its zenith until Day 3.5. This half-day delay in *Brachyury* expression goes hand-in-hand with the significant half-day delay in emergence of blast colonies.

2.4. The distribution of colonies in early hematopoiesis is altered

In the hematopoietic "big mix" assay of *Ctbp2*-B RNAi cultures, there are more total colonies but there is no significant difference in the absolute number of hematopoietic colonies (primitive erythroid, macrophage, erythroid-macrophage, and mixed lineage). There is a clear difference in the distribution of colonies in *Ctbp2*-B RNAi, with the number of secondary EBs dominating the total colony distribution and dwarfing the number of hematopoietic colonies (Supplementary materials, Fig. S4B). In addition to a relative deficiency in hematopoietic colonies, which characterize in vitro hematopoiesis, we also characterized a deficiency in *Scl* expression in *Ctbp2*-B RNAi (Supplementary materials, Fig. S4A).

3. Discussion

In this study, our goal was to explore the effect of loss of CtBP2, in vitro, in order to better characterize the observed in vivo defects that arise between E6.5–E10.5, prior to embryonic lethality. ESC assay results described herein provide an in vitro view of null embryonic development and demonstrate that although the defect in null mice is not fully visible until E10.5, there are important and progressive changes in the developmental program from the blastocyst to gastrulation

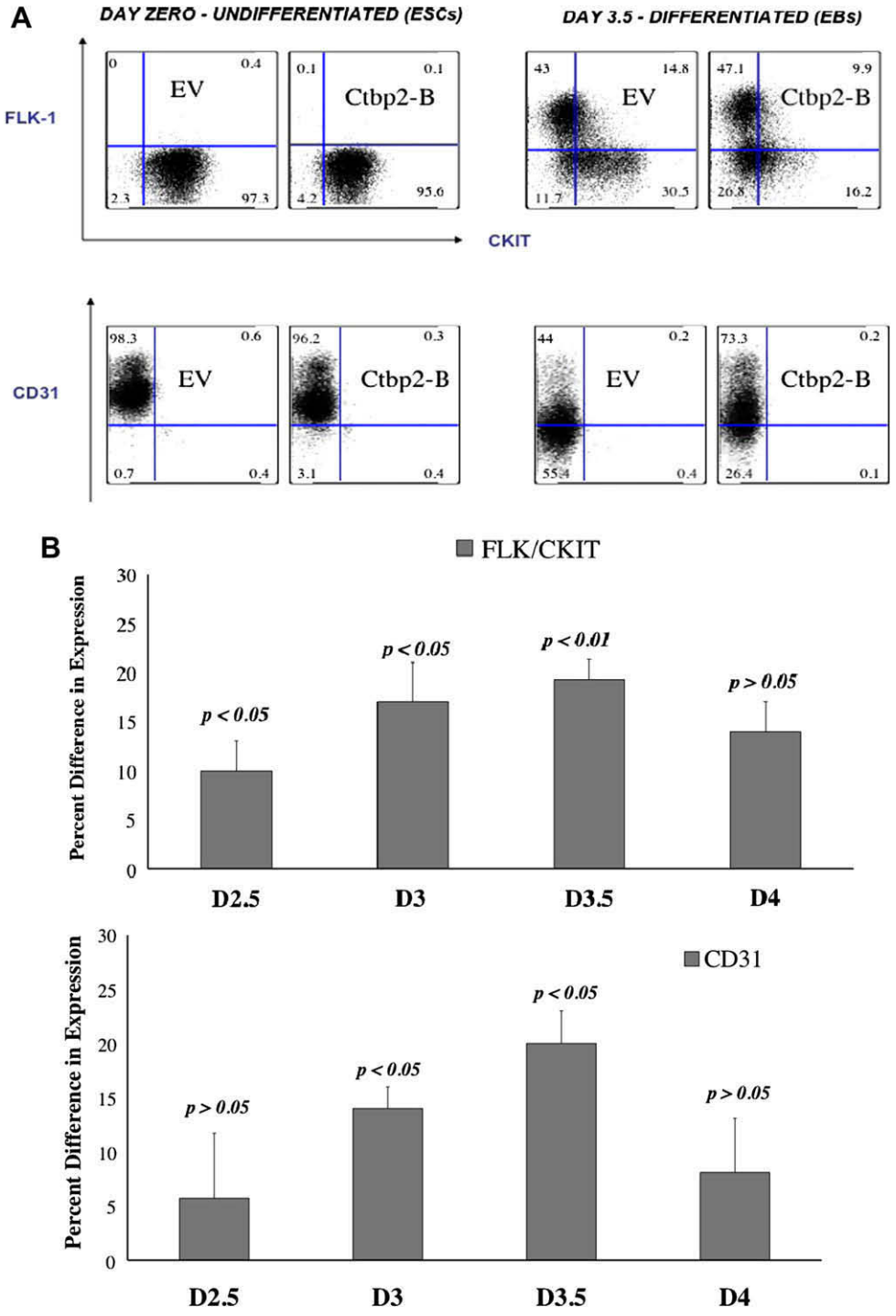

Fig. 4 – Delay in hemangioblast formation. (A) FACS profile of embryoid body (EB) differentiation in empty vector (EV) and *Ctbp2*-B RNAi cultures at Day 0 (left) and Day 3.5 (right). (B) Percent differences between *Ctbp2*-B RNAi EBs and EV RNA EBs in FLK-1/C-KIT (top) and CD31 (bottom) FACS expression at D2.5, D3, D3.5, and D4. Data represents the timepoint averages from three independent experiments.

stages. There are also important changes to the development of the hemangioblast. The hemangioblast does not represent the mesoderm, per se, but does represent the potential of the mesoderm to progress through development and give rise to differentiated cells.

This study focused primarily on characterizing *Ctbp2* knockdown culture development from ESC (blastocyst) to EB (gastrula) and then on the emergence and function of the blast colony (hemangioblast). A functional approach was used to characterize the effect of a loss of *Ctbp2* in ESC maintenance and in the development of mesodermal progenitors. Findings indicate that a knockdown of *Ctbp2* expression skews the balance between self-renewal and differentiation towards self-renewal even when differentiation is forced. A

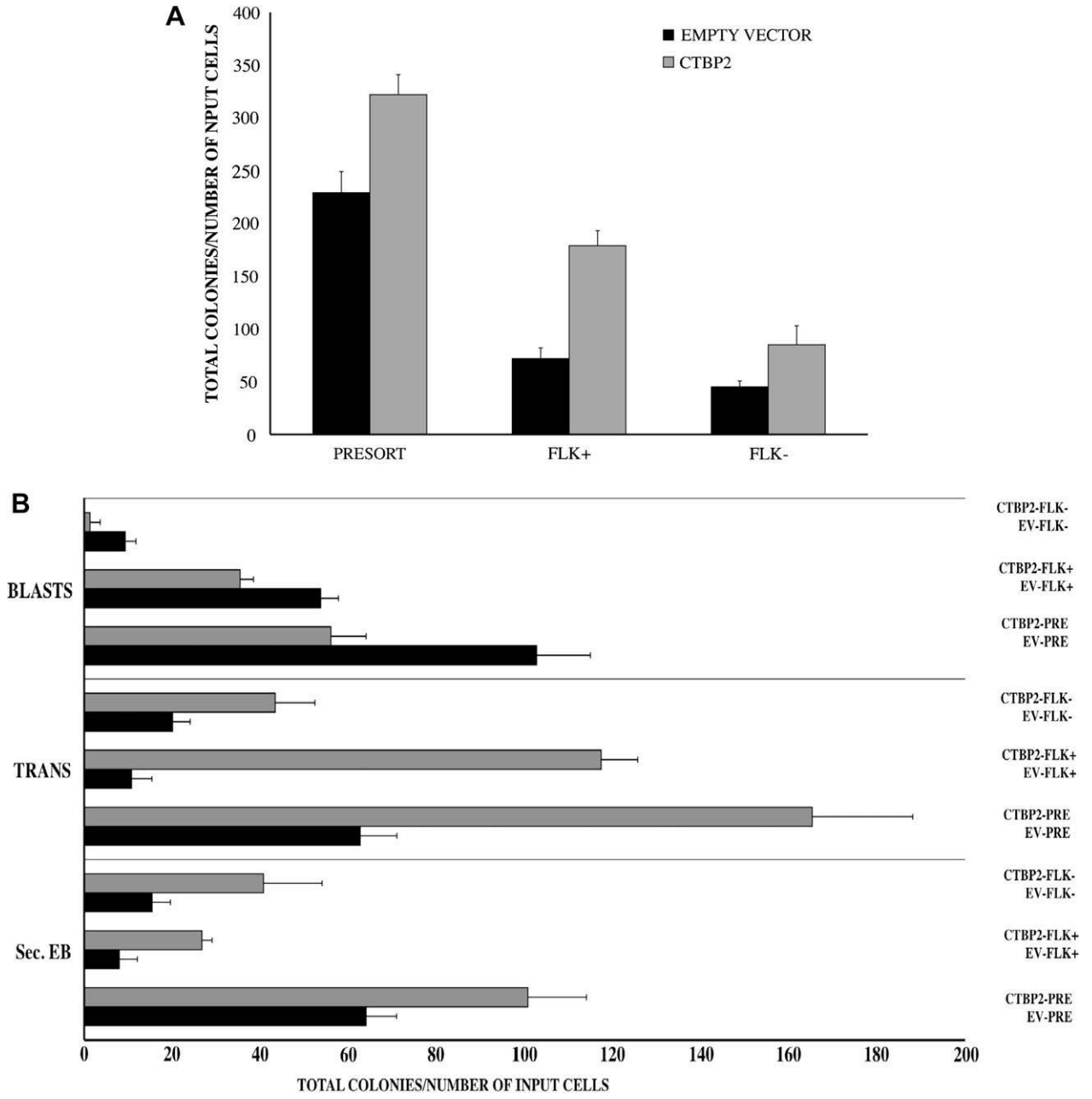

Fig. 5 – (A) Total number of blast assay colonies from a FLK+ population isolated from embryoid bodies (EBs) at Day 3.5. Data is representative of cultures in triplicate with $p < 0.05$. (B) Distribution of type of blast assay colony, generated from a FLK+ population isolated from EBs at Day 3.5, according to standard morphologies for secondary EB, transitional, or blast colonies. Empty vector (EV) is pictured in black and *Ctbp2*-B RNAi (*Ctbp2*) in grey. Cells from Day 3.5 EBs were isolated prior to sorting and according to GFP expression only (Pre), from sorting according to GFP and Flk-1 expression (FLK+), and from sorting according to GFP expression and the absence of Flk-1 expression (FLK−). Data is representative of cultures in triplicate with $p < 0.05$.

competition assay was first used to quantify an increase in prevalence in ESCs in the absence of *Ctbp2* and then cell cycle assays verified that this change is not attributable to changes in rate of cell division. Since there are only two mechanisms by which a stem cell could increase in prevalence, these being to divide more rapidly or to experience a change in symmetry of fate decisions, it was hypothesized that the latter mechanism was responsible for our observations. The hypothesis was supported by results from the AP assay in LIF− conditions and the quantification of *Oct4* expression in a differentiation timecourse. These results lead to the conclusion that *Ctbp2* is important for normal asymmetric differentiation. A decrease in CtBP2 creates a noticeable delay in ESC differentiation, but it does not completely block differentiation as evidenced by our ability to generate embryoid bodies. However, the embryoid bodies with knocked down *Ctbp2*

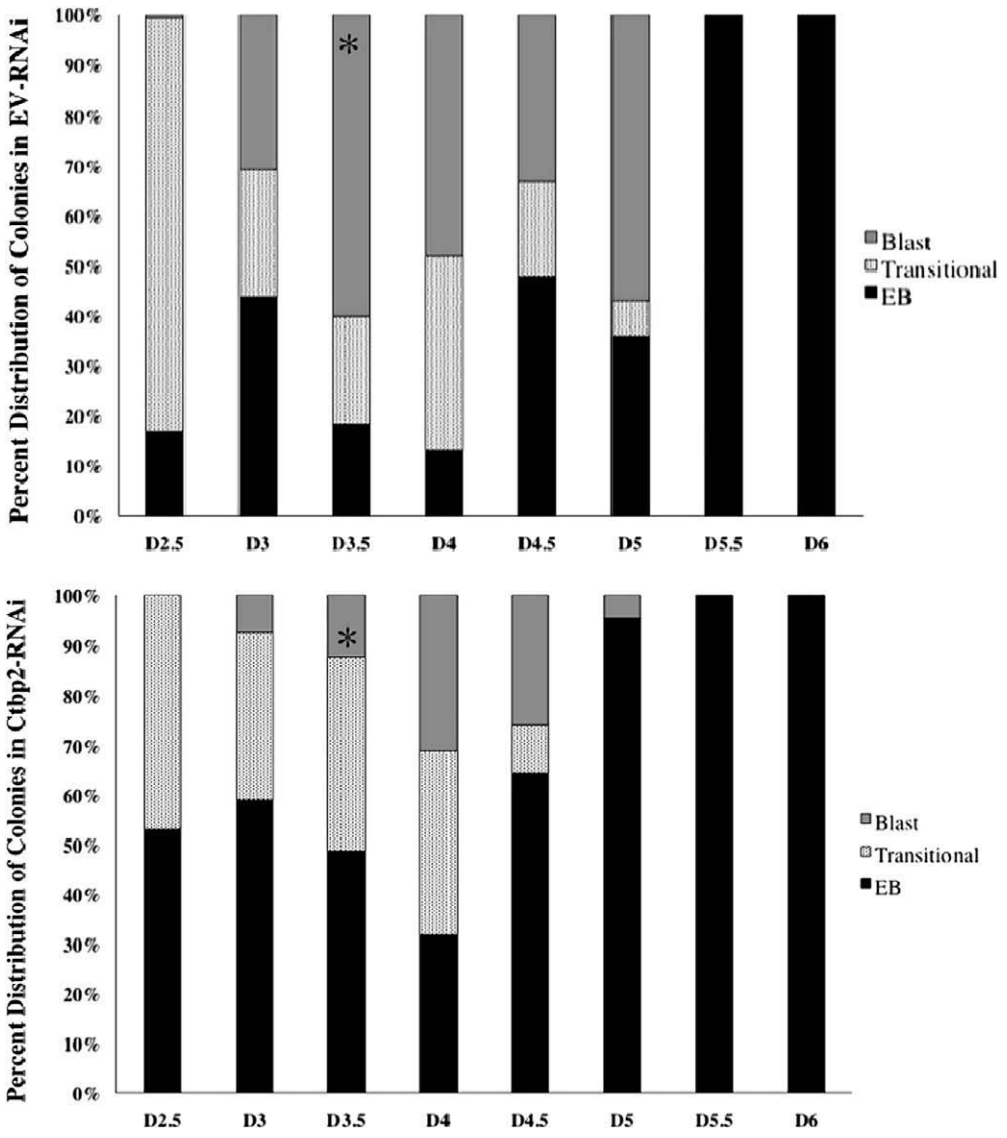

Fig. 6 – Distribution of secondary embryoid body (EB), transitional and blast colonies across Days 2.5–6. The significant difference ($p < 0.05$) in blast colonies between empty vector (EV, top) and *Ctbp2*-B RNAi (bottom) at D3.5 noted by asterisk (*). Distribution at each timepoint is representative of triplicate cultures. Data at D4 and D6 represents triplicate cultures in two independent experiments and data at D3 and D3.5 represents triplicate cultures in three independent experiments.

expression are not normal. Emergence of blast colonies from *Ctbp2*-B RNAi EBs is delayed. In vitro, the expectation is to isolate the highest number of mesodermal progenitor colonies, or blast colonies, from EBs that are at Day 3.5 of differentiation but in the absence of *Ctbp2*, peak blast formation is delayed until Day 4. The timing of this isolation is critical as primitive hematopoiesis rides on the coat tails of blast formation and what might appear to be a small delay of 12 h is significant for the rapidly developing embryo.

In addition to a delay in blast formation, there is a significant accumulation of immature blast colony precursors. If differentiation occurred normally, then the expectation is to isolate a larger number of differentiated blast colonies from *Ctbp2*-B RNAi. Transitionals, defined in Robertson et al. (2000) as colonies containing cell populations undergoing commitment from mesoderm to hematopoietic and endothelial lineages, should be in the minority by Day 3.5. Instead with *Ctbp2*-B RNAi, there are a proportionally smaller number of mature blast colonies and a larger number of immature transitional and secondary EB colonies. Additionally, if the blast colonies that emerged from *Ctbp2*-B RNAi EBs were normal, then in the hematopoietic assay the total number of primitive and definitive hematopoietic colonies should have greatly surpassed those of EV RNAi. Instead the numbers of hematopoietic colonies are comparable to EV RNAi but only make up 10% of the total colonies in *Ctbp2*-B RNAi.

Gene expression data and results of Western blots for signaling molecules can be used in conjunction with existing knowledge to build a mechanistic model for *Ctbp2* in embryonic development. In addition to characterizing functional delays in differentiation, this study found changes in *Oct4*, *Brachyury* and *Scl* expression. Abnormal changes in protein

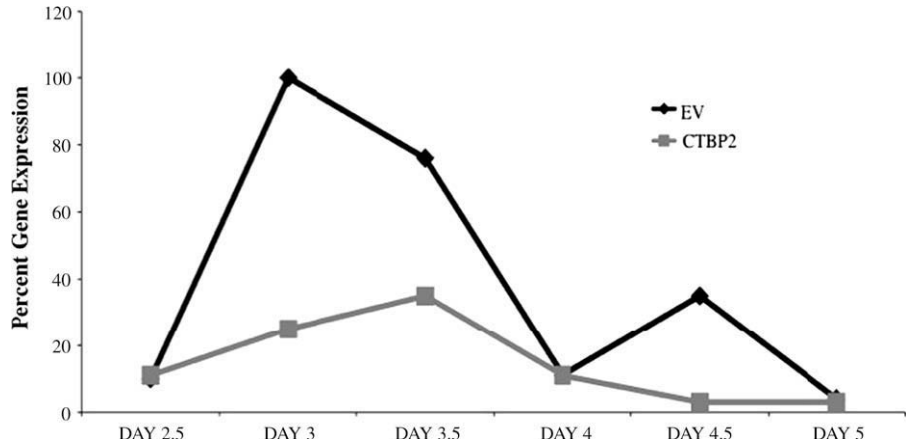

Fig. 7 – Expression of *Brachyury* in embryoid body (EB) differentiation. Quantified for empty vector (EV) and *Ctbp2*-B RNAi (*Ctbp2*) cultures using RT-PCR.

levels and phosphorylation were not evident in STAT3 or AKT signaling or in levels of phosphorylated GSK3-β and β-catenin. STAT3 is phosphorylated in response to an activating signal induced by the LIF ligand. We see normal STAT3 availability and phosphorylation in LIF+ conditions and the expected loss of phosphorylation in LIF− conditions. Although cultures lacking CtBP2 lose STAT3 phosphorylation upon LIF withdrawal, they retain *Oct4* expression well into induced differentiation. This retention of *Oct4* expression is attributed as the mechanism behind the *Ctbp2*-B RNAi cultures' self-renewal resiliency in the absence of LIF. Since CtBP2 is a transcriptional co-repressor, it would be worth exploring in future biochemical studies whether there is a physical binding of CtBP2 to the *Oct4* promoter or if CtBP2 is repressing *Oct4* by binding to other genetic elements or co-activators.

A recent study reports that STAT3 and AKT pathways make up a parallel circuit for maintaining self-renewal in ESCs in the absence of LIF (Niwa et al., 2009). We were able to exclude STAT3 signaling as a potential mechanism for the *Ctbp2*-B RNAi phenotype and in our analysis of AKT signaling in the presence of LIF, we do not observe a change in protein or phosphorylation levels. This study does not exclude the potential for an AKT-associated signaling mechanism in *Ctbp2*-B RNAi cultures in the absence of LIF (Supplementary materials, Fig. S1C). However, given our identification of both a gain of function phenotype in the presence of LIF and a downward trajectory of *Oct4* expression after LIF withdrawal, the signal regulating CtBP2's repressor activity should be apparent in both the presence and absence of LIF.

The phenotypes observed in the blast assays, namely a delay in blast colony emergence and an unusual abundance of transitional colonies, are attributed to a loss of *Brachyury* and *Scl*. These in vitro findings parallel those from in vivo embryos with defects in *Brachyury* expression and from *Scl*−/− EBs (Robertson et al., 2000). A decrease in *Brachyury* expression was noted in the *Ctbp2* null and linked to Wnt signaling, since CtBP2 is reported to function with β-catenin as a transcriptional activator of *Brachyury* (Yamaguchi et al., 1999; Hildebrand and Soriano, 2002). Our study does not find a change in GSK3-β or β-catenin levels in the absence of CtBP2, therefore, we can confirm that CtBP2 functions alongside β-catenin instead of functioning upstream of β-catenin. We can conclude that CtBP2 is required for normal transcription of *Brachyury* and that Wnt signaling alone is necessary but not sufficient to activate normal transcription of *Brachyury*. There are no published findings with regard to CtBP2 regulation of *Scl*; however, defects in yolk sac vascularization are noted in the *Ctbp2* null (Hildebrand and Soriano, 2002). *Scl* is recognized as a key regulator of hematopoietic specification and yolk sac angiogenesis, so the in vivo hematopoietic defects and the abundance of transitionals in vitro point toward a loss of *Scl* (Robertson et al., 2000; Lecuyer and Hoang, 2004). Gene expression analysis confirms a decrease in expression of *Scl* in *Ctbp2*-B RNAi and leads to the conclusion that *Ctbp2* is important in embryonic development as a transcriptional activator of *Scl*. Protein–protein interactions and promoter studies are still needed to characterize CtBP2's effect as a direct activator or as an indirect activator of *Scl* gene expression.

The decision to self-renew or differentiate is not as black and white as it might appear and this existence of a gray area enables ESCs to respond to the constantly changing demands of development. Pluripotency depends on an ability to balance fates and choose between symmetric self-renewal, asymmetric cell division, or commitment. The findings from this study demonstrate a clear role for *Ctbp2* in embryonic development. The model proposed is one in which CtBP2 normally represses *Oct4*, either directly or via a yet to be determined signaling pathway, to permit ESC differentiation and maintain asymmetric self-renewal. The model also proposes that CtBP2 directly activates transcription of *Brachyury* and *Scl* to permit normal blast formation and induction of hematopoiesis.

The proposed role of CtBP2 is to establish a pattern of gene expression that favors pluripotency and asymmetric self-renewal. The loss of CtBP2 does not completely block differentiation in ESC or blast assays and does not completely block transcription of *Brachyury* or *Scl*, which suggests the presence of a secondary transcriptional activator with similar function. From a developmental perspective, the presence of a secondary regulator in the absence of CtBP2 will only rescue the developing embryo if the activity occurs in parallel or if nor-

mal expression of *Brachyury* and *Scl* is maintained. Given the observed in vivo defects which lead to embryonic lethality and the delays in the in vitro assays that lead to aberrant ESC differentiation and blast formation, we infer that the secondary regulatory does not function in parallel to CtBP2, but rather, is activated in response to a loss of CtBP2. In sum, the *Ctbp2*-B RNAi cultures do eventually differentiate but unfortunately, with regard to embryonic development, delays in developmental cues are often lethal. In addition to continued characterization of biochemical interactions between CtBP2 and stem cell gene promoters to further our knowledge of the developmental regulation in the in vivo null, the finding of an abundance of undifferentiated progenitors also suggests that future studies include an analysis of *Ctbp2* in tumor models.

4. Experimental procedures

4.1. *Candidate selection and RNA interference design*

Ctbp2 was selected from microarray expression data sets based on differential expression (enrichment) in stem cells, in comparison to committed progenitors (Ivanova et al., 2002; Ramalho-Santos, 2002). The criteria used were identification as a chromatin factor and a greater than twofold level of enrichment in stem cells. *Ctbp2* satisfied both criteria sufficiently as a chromatin-related co-repressor of transcription with a level of expression in mouse embryonic stem cells five times that measured in committed progenitors (Ivanova et al., 2002).

Dharmacon's RNAi design program (http://www.dharmacon.com/sidesign) was chosen based on specificity in the design of siRNA sequences and use of the National Institutes of Health (NIH) Blast algorithm to exclude sequences with any homology to *Ctbp2*. Blast of *Ctbp1* and *Ctbp2* shows 76% sequence homology. Blast of the siRNA sequence *Ctbp2*-A to the coding sequence of *Ctbp2* shows pairing of all 19 nucleotides at position 1924–1942. There are six additional pairings possible but the largest of these is only 8/19 nucleotides. Blast of the siRNA sequence *Ctbp2*-A to the coding sequence of *Ctbp1* shows the largest match as only 9/19 nucleotides, with an additional seven pairings of no more than 8/19 nucleotides. Blast of the siRNA sequence of *Ctbp2*-B to the coding sequence of *Ctbp2* shows pairing of all 19 nucleotides at position 2594–2612. There are an additional four pairings possible but the largest match is only 9/19 nucleotides. Blast of the siRNA sequence *Ctbp2*-B to the coding sequence of *Ctbp1* shows that there is only one possible pairing of 7/19 nucleotides.

Some publications have suggested an effect of the anti-sense strand of the siRNA construct, so to be cautious, we blasted this sequence as well. Our findings show that the anti-sense sequence of *Ctbp2* construct A with the *Ctbp2* coding sequence yields one match of 7/19 while a blast of the same construct with the *Ctbp1* coding sequence yields three pairings, all of 8/19 base pairings or less. The anti-sense sequence of *Ctbp2* construct B blasted against the *Ctbp2* coding sequence produces six matches of 8/19 base pairings or less, and a blast of the same construct with the *Ctbp1* coding sequence yields only one match of 7/19 base pairings. The conventional minimum threshold for bioinformatics exclusion is less than 15 base pairs of homology. The homology of the additional pairings shown here is far below that threshold. Jackson et al. (2003) tested the thresholds at which siRNA becomes ineffective and their findings affirm 15 base pairs of homology as the threshold.

4.2. *Lentiviral vector production and RT-PCR for gene expression*

A third generation lentiviral vector FUGW (Lois et al., 2002) was modified as described in Ivanova et al. (2006) to express a short-hairpin RNA (shRNA) from an H1 promoter. Sequence-specific RNAi cassettes were designed in part using Dharmacon's siRNA Design Center. The Dharmacon software was used to, first, identify a gene specific target within the open reading frame (ORF) and, second, to generate a 19 base, sequence-specific oligonucleotide. Two shRNA cassettes were generated per gene target. The cassette that targeted the more 5′ region of the ORF was labeled "A" and the cassette that targeted the more 3′ region of the ORF was labeled "B". The oligonucleotides generated are as follows: *Ctbp2*-A (ATTC GACAGTCTGCCACAT) and *Ctbp2*-B (AGAATTCTTCGTGACT TCA). The shRNA cassettes were cloned directionally into the vector using XbaI and SmaI cloning sites. Cloning was verified by restriction digestion with PacI and sequencing of the fragment product with a primer for the HIV-1 flap (ACAGCAGAGATCCAGTTTG). Each sequence-verified shRNA lentiviral plasmid was co-transfected with pVSV-G and pCMVd8.9 packaging plasmids into 293FT cells. At 16–22 h prior to co-transfection, the 293FT cells were plated on pregelatinized 15 cm dishes at 18×10^6 cells per dish and cultured at 37 °C. At transfection, 3–15 cm dishes of cultured 293FT cells were used per lentiviral construct. Superfect was used to mediate transfection and the transfected 293FT cells were cultured at 37 °C for 20–24 h and moved to 32 °C for an additional 40–44 h of culture. The viral supernatant was harvested from the dishes, filtered through a 0.44 μm low-protein binding filter, and centrifuged for 150 min at 50,000*g*. The resulting pellet was resuspended in 300 μL sterile PBS, aliquoted, and stored at −80 °C until use. Viral titers were measured using serial dilutions of viral construct to transduce NIH 3T3 fibroblasts and then FACS analysis measured expression of GFP to confirm transduction efficiency. For quantification of RNAi efficiency in gene expression knockdown, all transduced ESCs were sorted by FACS, according to expression of GFP, into Trizol for RNA extraction and cDNA synthesis. Quantitative polymerase chain reaction (Q-PCR) was conducted on cDNA samples from cells sorted on Days 4, 8, 11 and 12 post-transduction (p.t.).

4.3. *Cell culture*

CCE murine ES cells (Advanced Cell Technologies) were maintained on gelatinized plates in self-renewal media (DMEM supplemented with 10% FCS, glutamine, non-essential amino acids, sodium pyruvate, BME, P/S, and LIF). J1 murine ES cells (a gift from Dr. Gordon Keller, Mt. Sinai School of Medicine) were cultured on irradiated mouse primary embryonic feeders as reported (D'Souza et al., 2005). Transductions were performed as follows: CCE ES cells were trypsinized

and resuspended to a concentration of 400,000 cells/mL in CCE self-renewal media supplemented with polybrien. For each vector, 1 mL of cells was mixed with the lentiviral RNAi construct at low multiplicity of infection (MOI of 5), plated in one well of a pre-gelatinized 6-well dish, and cultured at 37 °C for 20–24 h. After the first 20–24 h period, 1 mL of new CCE self-renewal media was added to each well of the dish and the dish was cultured for an additional 24 h to maximize GFP expression from the lentiviral construct. J1 cells were transduced in a similar fashion except for the inclusion of a feeder depletion step, on an ungelatinized dish for 1–1.5 h, prior to the addition of lentiviral RNAi construct.

4.4. *Quantitative and semi-quantitative PCR*

Cells were sorted into Trizol at a concentration of 100,000 cells/mL. Total RNA was extracted using a standard RNA isolation protocol. Total RNA was transcribed into cDNA with random hexamers using Powerscript. Semi-quantitative PCR and Q-PCR were carried out using gene-specific primers.

Gapdh	TCCCACTCTTCCACCTTCGATGC	Forward
	GGGTCTGGGATGGAAATTGTGAGG	Reverse
Ctbp2	ACCACACACAGCCTGGTACA	Forward
	CCTGGAGCCACACCTACAAT	Reverse
Oct4	GCAGGAGCACGAGTGGAAAGCAAC	Forward
	CAAGGCCTCGAAGCGACAGATG	Reverse
Brachyury	CATGTACTCTTTCTTGCTGG	Forward
	GGTCTCGGGAAAGCAGTGGC	Reverse
Scl	CCAGCAAGCTGAGGAGCGGCG	Forward
	CGGATTTGTAAAGACGGTGA	Reverse

4.5. *Immunochemistry*

For Western blotting, whole cell proteins were isolated with RIPA buffer and nuclear proteins were isolated with TMSB buffer (25 mM NaCl, 10 mM Tris–HCl at pH 7.8, 1.5 mM $MgCl_2$, 0.25 M sucrose, 10 mM BME, 0.5 mM PMSF) and F buffer (10 mM Tris, pH 7.05, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 μM $ZnCl_2$, 0.1 mM Na_3VO_4, 10% glycerol, 0.1% Triton X-100, aprotinin, PMSF). After SDS–PAGE, the nitrocellulose was blotted with antibodies to CtBP2 (Santa Cruz #5967), Phospho-STAT3 (Cell Signaling Technologies #9138), STAT3 (Cell Signaling Technologies #9139), Actin (Sigma #A2066), SMARCA4 (Upstate #07-478), Phospho-AKT Pathway Sampler kit (Cell Signaling Technologies #9916), MAPK/ERK-1/2 (Cell Signaling Technologies #9102), β-catenin (a gift from Dr. Kateri Moore, Princeton University), and ERK-1/2 (a gift from Dr. Jean Schwarzbauer, Princeton University).

4.6. *Competition and proliferation assays*

Transduced cells were sorted on a FACS Vantage according to GFP expression. GFP-positive cells were plated into competitive assays, in a 6-well dish at 37 °C as described in detail in Ivanova et al., 2006. Cultures were analyzed for GFP expression by FACS at each 48-h passage. Briefly, the ESC competition assay has been used in previously published studies to assess self-renewal ability (Ivanova et al., 2006; Galan-Caridad et al., 2007) and is based on the classic hematopoietic stem cell (HSC) competitive repopulating assay. Normal stem cells, GFP−, set the threshold for self-renewal and differentiation activity in culture. Mutant ESCs, GFP+, are placed in self-renewal competition with the normal ESCs. The ratio used for most competition assays is 50% normal ESCs and 50% mutant ESCs. If the mutation or knockdown in the mutant ESCs has no affect on self-renewal ability, then the ratio of 50/50, as assayed by FACS for GFP−/GFP+, will be maintained over the course of the culture period (+). If the mutation leads to a loss of ESC self-renewal ability, then the mutant ESCs will differentiate and will not be included in the GFP+ stem cell category. Differentiated ESCs are larger and can be separated out from undifferentiated ESCs according to FACS scatter profile. The resulting ratio will favor a higher percentage of normal stem cells (GFP−) as the dominant cell type in the culture. If the mutation leads to a gain of self-renewal ability above what is normally observed in normal cultures, then the ratio of GFP−/GFP+ ESCs will shift towards the GFP+ cell population. We used internal controls to ensure that the difference in GFP−/GFP+ is not attributable to transfection reagents, handling (mock transfection) or to RNAi method (empty vector). We also used a positive control for loss of self-renewal (*Oct4* RNAi).

To ascertain rate of proliferation, cultures were first analyzed using PKH26 dye retention (Sigma) and Annexin V staining (Roche) according to manufacturer's instructions to exclude the likelihood of abnormal changes in cell cycle or rate of apoptosis. Afterwards, control and knockdown cells were grown in separate dishes with cell counts at passage, every 36-h, for four passages. For each passage, we first counted the cell number in culture for the previous passage and then we replated a specific number of cells (identical number for both control and knockdown cultures) so that each passage began with the same cell number per culture dish.

4.7. *Differentiation*

For the alkaline phosphatase (AP) assay, ESCs were sorted at Day 2 post-transduction and plated in LIF+ or LIF− conditions in DMEM supplemented with 10% FCS (tested for ESCs), glutamine, non-essential amino acids, sodium pyruvate, BME, and P/S as reported (Ivanova et al., 2006). Cultures were stained and assayed using an AP kit (Sigma) at Day 9 post-transduction and colony size was measured with IP Lab (Scanalytics Inc.) software.

For embryoid body (EB) differentiation, ESCs were differentiated under EB-generating conditions in DMEM, 10% FCS (tested for differentiation), glutamine, non-essential amino acids, sodium pyruvate, BME, and P/S. EBs were trypsinized and dissociated prior to analysis by FACS with antibodies

against FLK-1 (a gift from Dr. Gordon Keller, Mt. Sinai), PE-conjugated C-KIT (BD Pharmingen #553355), and biotin-conjugated CD31 (BD Pharmingen #553371). Blast assay colonies and hematopoietic "big mix" colonies were isolated and scored using published methods (Choi et al., 1998; Fehling et al., 2003; Kubo et al., 2004; D'Souza et al., 2005; Ivanova et al., 2006).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2009.10.002.

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