TRIM28 is required by the mouse KRAB domain protein ZFP568 to control convergent extension and morphogenesis of extra-embryonic tissues

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SUMMARY

TRIM28 is a transcriptional regulator that is essential for embryonic development and is implicated in a variety of human diseases. The roles of TRIM28 in distinct biological processes are thought to depend on its interaction with factors that determine its DNA target specificity. However, functional evidence linking TRIM28 to specific co-factors is scarce. chatwo, a hypomorphic allele of Trim28, causes embryonic lethality and defects in convergent extension and morphogenesis of extra-embryonic tissues. These phenotypes are remarkably similar to those of mutants in the Krüppel-associated box (KRAB) zinc finger protein ZFP568, providing strong genetic evidence that ZFP568 and TRIM28 control morphogenesis through a common molecular mechanism. We determined that chatwo mutations decrease TRIM28 protein stability and repressive activity, disrupting both ZFP568-dependent and ZFP568-independent roles of TRIM28. These results, together with the analysis of embryos bearing a conditional inactivation of Trim28 in embryonic-derived tissues, revealed that TRIM28 is differentially required by ZFP568 and other factors during the early stages of mouse embryogenesis. In addition to uncovering novel roles of TRIM28 in convergent extension and morphogenesis of extra-embryonic tissues, our characterization of chatwo mutants demonstrates that KRAB domain proteins are essential to determine some of the biological functions of TRIM28.

KEY WORDS: KAP1, KRAB, Convergent extension, Extra-embryonic tissues, Gastrulation, Mouse

INTRODUCTION

Tripartite motif protein 28 (TRIM28), also known as KRAB-associated protein 1 (KAP1), KRAB interacting protein 1 (KRIPI), or transcription intermediary factor 1 β (TIF1β), encodes a TRIM/RBCC motif (RING finger, B box, coiled coil), plant homeodomain (PHD) finger and bromodomain protein that functions as a strong transcriptional repressor when bound to DNA (Friedman et al., 1996; Kim et al., 1996; Moosmann et al., 1996). The ability of TRIM28 to repress transcription has been proposed to reside in its ability to recruit chromatin-modifying enzymes, including the SETDB1 histone 3 lysine 9 methyltransferase (Schultz et al., 2002) and CHD3, a component of the nucleosome remodeling and histone deacetylation (NuRD) complex (Schultz et al., 2001). TRIM28 also binds heterochromatin protein 1 (Nielsen et al., 1999; Ryan et al., 1999), an interaction that affects TRIM28 localization to heterochromatic regions (Cammas et al., 2002), as well as some TRIM28 biological functions (Cammas et al., 2004; Herzog et al., 2010). In addition to its well-documented roles as a transcriptional repressor, TRIM28 has recently been proposed to activate transcription through its ability to bind transcription factors, such as OCT3/4 (POU5F1 – Mouse Genome Informatics) (Seki et al., 2010), NGFI-B (NR4A1 – Mouse Genome Informatics) (Rambaud et al., 2009) and C/EBPβ (CEBPB – Mouse Genome Informatics) (Chang et al., 1998), raising the possibility that the formation of multimeric complexes with other proteins can modulate TRIM28 transcriptional activity.

Trim28 knockout mouse mutants fail to gastrulate and they die at embryonic day (E) 5.5 (Cammas et al., 2000). Additionally, TRIM28 has essential roles in a broad range of biological processes including spermatogenesis (Weber et al., 2002), silencing of endogenous retroviral elements (Rowe et al., 2010; Wolf et al., 2008a; Wolf and Goff, 2007; Wolf et al., 2008b), maintenance of embryonic stem (ES) cell pluripotency (Hu et al., 2009; Seki et al., 2010), epigenetic phenotypic variation (Whitelaw et al., 2010), cancer metastasis (Ho et al., 2009; Yokoe et al., 2009) and anxiety disorders (Alter and Hen, 2008; Jakobsson et al., 2008). Even though the transcriptional targets and co-factors of TRIM28 involved in the control of these biological processes are largely unknown, the DNA-binding specificity of TRIM28 is believed to be provided through its interaction with proteins of the Krüppel-associated box (KRAB) zinc finger protein family (Urrutia, 2003), a large family of transcription factors found exclusively in tetrapod vertebrates (Agata et al., 1999; Gebelein and Urrutia, 2001; Iyengar et al., 2011; Moosmann et al., 1996; Urrutia, 2003). Proteins of this family contain a KRAB domain, which mediates interaction with the TRIM28 N-terminal RBCC domain (Germain-Desprez et al., 2003; Peng et al., 2000; Peng et al., 2002) and a variable number of zinc finger motifs, which are thought to provide DNA-binding specificity to different targets (Emerson and Thomas, 2009; Gebelein and Urrutia, 2001). On the basis of its ability to enhance KRAB-mediated transcriptional repression, TRIM28 has been proposed to function as the universal co-repressor of all KRAB domain-containing proteins (Abrink et al., 2001; Agata et al., 1999; Friedman et al., 1996; Moosmann et al., 1996). Although KRAB domain zinc fingers represent the largest family of transcription...
factors in mammals, comprising more than 300 genes (Emerson and Thomas, 2009; Huntley et al., 2006; Rowe et al., 2010), our current knowledge about the roles of individual KRAB domain proteins is limited to just a handful of these factors: ZFP568 is essential for embryo morphogenesis (Garcia-Garcia et al., 2008; Shibata and Garcia-Garcia, 2011), ZFP57 is required for the establishment and maintenance of genomic imprinting (Li et al., 2008), ZFP809 has been involved in silencing of retroviral elements in ES cells (Wolf and Goff, 2009), RSL1 and RSL2 regulate sex-specific gene expression in the liver (Krebs et al., 2003) and ZNF746 has been linked to parkin-dependent neurodegeneration (Shin et al., 2011). Even though several studies have demonstrated a functional link between some of these KRAB zinc finger proteins and TRIM28 (Gebelein and Urrutia, 2001; Li et al., 2008; Wolf and Goff, 2009), evidence supporting a role of TRIM28 as the universal co-repressor of all KRAB domain proteins is limited to just a handful of these factors: ZFP568 is widely and dynamically expressed during the early stages of mouse embryogenesis (Garcia-Garcia et al., 2008; Shibata and Garcia-Garcia, 2011). However, analysis of Zfp568 chimeric embryos revealed that Zfp568 is required in embryonic-derived cells to control morphogenesis of both embryonic and extra-embryonic tissues (Shibata and Garcia-Garcia, 2011). Here, we show that chatwo, an N-ethyl-N-nitosourea (ENU)-induced mutation that causes a similar phenotype to Zfp568 mutants, is a hypomorphic allele of Trim28. Our comparative analysis of Trim28chato and Zfp568chato mutant phenotypes provides strong evidence that ZFP568 and TRIM28 control morphogenesis of embryonic tissues through a common molecular mechanism. Consistent with this, we found that TRIM28 binds to ZFP568 and is required to mediate ZFP568 transcriptional repression. We found that chatwo mutations affect TRIM28 protein stability and repressive activity, disrupting both ZFP568-dependent and ZFP568-independent TRIM28 functions. Together with the analysis of null Trim28KO mutants and embryos bearing a conditional inactivation of Trim28 in embryonic-derived tissues, our results demonstrate that TRIM28 is indispensable for ZFP568 activity during embryo morphogenesis, and that TRIM28 is differentially required by ZFP568 and other factors in a tissue-specific manner. Our results uncover novel roles of TRIM28 during early mouse embryogenesis and provide mechanistic insight into the functions of TRIM28 as a co-factor of KRAB domain proteins.

**MATERIALS AND METHODS**

**Mouse strains**

chatwo was characterized on FvB/NJ and C57BL/6 Mls musculus strain backgrounds. Phenotype expressivity was quantified in embryos from congenic FvB/NJ and N7 backcrossed C57BL/6 animals. D7Mit178 and D7Mit76 polymorphic markers were used for genotyping. Zfp568chato (Garcia-Garcia et al., 2008), Trim28 knockout and Trim282/2 conditional mice (Cammas et al., 2000) were previously described. Sox2Cre mice were obtained from Jackson Laboratory (Hayashi et al., 2002). Sox2Cre; Trim282/2KO or Trim282/2;chatwo females were crossed into Sox2Cre; Trim282/2KO males. Experiments with mice were carried out in accordance with institutional and national regulations.

**Positional cloning of chatwo**

The chatwo mutation was created on a C57BL/6j genetic background, and outcrossed to FVB as described (Liem et al., 2009). Using a whole genome single nucleotide polymorphism (SNP) panel (Moran et al., 2006), the chatwo mutation was mapped to a 20 Mb region on proximal chromosome 7. Further mapping of meiotic recombinants narrowed the interval to SNPs rs31712695 and rs31644455. Trim28 cDNA was sequenced as amplified (Superscript One-Step RT-PCR, Invitrogen) from wild-type and chatwo RNA extracted at E8.5 (RNA STAT-60, Tel-Test).

**Embryo analysis**

Embryos were dissected in 4% bovine serum albumin (BSA) in PBS. In situ hybridizations were conducted as previously described (Shibata and Garcia-Garcia, 2011). The Trim28 probe was synthesized from a PCR-amplified cDNA fragment. Embryos were imaged in methanol, and cryosectioned at a thickness of 16 μm. Immunohistochemistry was performed as described (Nagy, 2003) on 8 μm cryosections. All comparisons of wild-type and mutant embryos are at the same magnification unless otherwise noted. Whole-mount embryos and sagittal sections are shown with anterior to the left and extra-embryonic tissues up. For western blotting, embryos were dissected in PBS and protein levels were quantified using Photoshop and linear regression analysis.

Expression of Trim28 and intracisternal A-type particle (IAP) elements was tested by qRT-PCR on RNA samples extracted from independent pools of either wild-type, Zfp568chato, Trim28chato and/or Trim282/2 embryos collected at E7.5 or E8.5 (RNA STAT-60, Tel-Test). SYBR Green real-time PCR was used to quantify cDNA samples synthesized using Superscript III First-Strand Synthesis (Invitrogen). Expression of IAP elements was tested on DNasel-treated (Roche) RNA samples. The absence of contaminating genomic DNA was confirmed by performing the assay in absence of reverse transcriptase.

**Yeast two-hybrid assays**

Gal4DBD and AD fusion plasmids were sequentially transformed into AH109 yeast strain using Matchmaker GAL4 Two-Hybrid System 3 (Clontech). Colonies were re-plated onto Ade-His-Leu-Trp- or Leu-His-Trp-X-alpha-gal plates.

**Cell culture**

HEK293, HEK293T or NIH3T3 cells were transfected with FUGENE 6 (Roche) or Lipofectamine 2000 (Invitrogen). A Leica DMI6000B fluorescent microscope was used for imaging. For immunoprecipitation, cells were lysed in buffer containing 150 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 1% Triton X-100, 0.05% SDS and protease inhibitors. Immunoprecipitations were performed using 2-3 μl of antibody and 25 μl protein A/G agarose beads (Santa Cruz Biotechnology). For luciferase assays, HEK293T cells were transfected with pGL35XUAS firefly luciferase reporter, a Gal4DBD effector and pRL Renilla luciferase plasmids. Total amount of DNA transfected was held constant by co-transfecting pCMV-MYC as needed. Cells were assayed with the Dual-Luciferase Reporter System (Promega) 24 hours after transfection. For each luciferase assay, duplicate transfections and replicate lysates were measured for each condition (n=4). Firefly luciferase expression was normalized to Renilla to control for transfection efficiency. Percent luciferase expression was calculated compared with Gal4DBD. Lysates loaded for western blotting were normalized to Renilla expression. Statistical analysis was performed using paired, two-tailed t-test.

**Reticulocyte translation assays**

Translation was assayed using the TNT Coupled Reticulocyte Lysate and Transcend Non-Radioactive Translation Detection Systems (Promega) in the presence of 1 μg plasmid DNA and 1 μl of transfected tRNA (biotinylated lysine). Translated protein was visualized by western blotting using Streptavidin-HRP (1:10,000).
Antibodies
The following antibodies were used for western blotting, co-immunoprecipitation (co-IP) and/or immunofluorescence: anti-TRIM28 (4E6, Sigma-Aldrich; 1:500), anti-TRIM28 (H-300, Santa Cruz Biotechnology; 1:500), anti-TRIM28 (MA1-2032, Thermo Scientific; 1:25), anti-CHD3 (Abcam; 1:700), anti-SETDB1 (Millipore; 1:1000), anti-GAL4DBD (RK5C1, Santa Cruz Biotechnology; 1:500-1:800), anti-Myc (9e10, Hybridoma Bank; 1:250-1:1000), anti-Flag (M2, Sigma-Aldrich; 1:500-1:700), anti-HA (11, Covance 1:250), anti-HA (Y11, Santa Cruz Biotechnology; 1:500), anti-GAPDH (AB9482, Abcam; 1:8000), antimouse/rabbit HRP (Jackson ImmunoResearch; 1:10,000), anti-PECAM (eBioscience; 1:200), anti-rat Alexa 488 (Molecular Probes; 1:200), antimouse Alexa 568 (Molecular Probes; 1:200).

Constructs and primers
Plasmids pCDNA3.1-Gal4DBD-TRIM28, pCDNA3.1-Gal4DBD-TRIM28ΔK5, pGL35XUAS firefly luciferase and pRL Renilla luciferase are described in Mascle et al. (Mascle et al., 2007). Other constructs were generated using primers as indicated in supplementary material Table S1.

RESULTS
chatwo causes embryonic and extra-embryonic defects similar to Zfp568 mutants
chatwo mutants were isolated in an ENU mutagenesis screen for recessive mutations affecting development of the mid-gestation mouse embryo. Embryos homozygous for chatwo arrested prior to E9 with severe convergent extension defects and disrupted morphogenesis of extra-embryonic tissues (Fig. 1). chatwo mutants had a short anterior-posterior axis and failed to undergo gut closure, giving chatwo embryos a characteristic U-shape (Fig. 1A-D). Additionally, extra-embryonic tissues in chatwo mutants appeared constricted and the yolk sac developed numerous bubble-like protrusions (Fig. 1C,D, arrowheads). These phenotypes were remarkably similar to those of chato mutants, which are homoyzogous for a null allele of Zfp568 (Fig. 1E,F) (Garcia-Garcia et al., 2008; Shibata and Garcia-Garcia, 2011), hence the name chatwo (cha-two; a second version of chato).

chatwo is a hypomorphic allele of Trim28
The chatwo mutation was mapped to a genetic interval on mouse chromosome 7 containing 9 genes (Fig. 2A). Trim28 stood out amongst these candidates, given its previous involvement as a co-repressor of KRAB domain proteins (Abrink et al., 2001; Agata et al., 1999; Friedman et al., 1996; Mooßmann et al., 1996), a large family of transcriptional regulators that includes ZFP568 (Emerson and Thomas, 2009; Huntley et al., 2006). Sequencing Trim28 in chatwo mutants revealed two adjacent point mutations in the Trim28 open reading frame (ORF), which respectively caused Cys713Trp and His714Asn non-conservative amino acid changes (Fig. 2B). These amino acid residues are highly conserved in human and Xenopus Trim28, as well as in other members of the TIF1 protein family (Fig. 2D). The chatwo mutations in Trim28 created a BstI restriction site that was used to confirm linkage of these sequence changes to the chatwo phenotype (Fig. 2C).

Trim28-null embryos (Trim28KO) arrest at ~E5.5, fail to gastrulate and lack expression of brachyury, a marker of the primitive streak (Fig. 2F) (Cammas et al., 2000; Wilkinson et al., 1990). We found that the chatwo allele had variable expressivity and sometimes caused developmental arrest at E7.5 (37.7%, n=182; supplementary material Fig. S1). However, the phenotype and stage of lethality of Trim28chatwo mutants were always different to those of Trim28KO embryos, as even chatwo embryos with an early developmental arrest expressed brachyury and were able to gastrulate (supplementary material Fig. S1C). These observations suggested that Trim28chatwo could be a hypomorphic allele. To obtain genetic confirmation that chatwo disrupts Trim28, we analyzed Trim28chatwoKO embryos. Like Trim28chatwo mutants, some Trim28chatwoKO embryos arrested at E7.5 (63.6%, n=7/11; Fig. 2H), whereas others survived until E8.5 (36.4%, n=4/11; Fig. 2G). Brachyury was expressed in all Trim28chatwoKO embryos examined, regardless of their stage of lethality, indicating that phenotypes of Trim28chatwoKO embryos are milder than those of Trim28KO mutants (Fig. 2E-H). These results support the hypothesis that chatwo mutations create a hypomorphic allele of Trim28. Additionally, the early lethality of Trim28KO mice (Cammas et al., 2000) compared with that of Trim28chatwo mutants and the null Zfp568chatwo embryos suggests that TRIM28 is required during the early stages of mouse morphogenesis for processes other than those controlled by ZFP568.

chatwo affects ZFP568-dependent and ZFP568-independent roles of TRIM28
We showed previously that Zfp568chatwo embryos have strong convergent extension defects, including failure to undergo anterior-posterior axis elongation, mediolateral expansion of mesenchymal
and epithelial tissues and an open neural tube (Garcia-Garcia et al., 2008). Phenotypic analysis confirmed that the phenotypes of Zfp568^chatwo^ embryos and Trim28^KO^ embryos with a late lethality were very similar. Analysis of Foxf1 expression, which marks the lateral plate mesoderm (Mahlapuu et al., 2001), showed that this tissue was shorter and wider in late-lethality Trim28^KO^ mutants than in wild-type littermates (Fig. 1A,C, brackets), and was located further away from the midline (Fig. 1G,H, dashed line). Expression of Meox1 (Candia et al., 1992) and Twist (Quertermous et al., 1994), showed that the somitic mesoderm was also mediolaterally expanded in late-lethality Trim28^KO^ embryos (not shown).

Additionally, late-lethality Trim28^KO^ mutants failed to close the neural tube (Fig. 1G,H). Expression of transthyretin (Ttr) (Cereghini et al., 1992), which labels visceral endoderm but not definitive endoderm, was used previously to evaluate convergent extension defects in Zfp568^KO^ mutants (Garcia-Garcia et al., 2008). We found that in late-lethality Trim28^KO^ embryos the definitive endoderm (Ttr-negative) failed to narrow (supplementary material Fig. S2E,G) to the same extent as Zfp568^KO^ embryos (Garcia-Garcia et al., 2008). Altogether, our analysis shows that the embryonic defects in late-lethality Trim28^KO^ embryos strongly resemble defects in Zfp568^KO^ embryos (Fig. 1E,F) (Garcia-Garcia et al., 2008).

Analysis of molecular markers in extra-embryonic tissues also highlighted similarities in the extra-embryonic phenotypes of Zfp568^KO^ mutants and late-lethality Trim28^KO^ embryos, although some phenotypic differences were notable. Like Zfp568^KO^ embryos, the yolk sac of Trim28^KO^ mutants had numerous bubble-like protrusions (Fig. 1C,D, arrowheads). However, yolk sac blisters in late-lethality Trim28^KO^ embryos were found throughout the entire yolk sac, whereas they often clustered in a region proximal to the embryo in Zfp568^KO^ mutants (Fig. 1C-F) (Shibata and Garcia-Garcia, 2011). In Zfp568^KO^ embryos, defects in placental morphogenesis originate from the failure of the allantois to extend and contact the chorion, as well as from an expansion of the chorionic trophoderm and failure of the ectoplacental cavity to collapse (Shibata and Garcia-Garcia, 2011). Similar to Zfp568^KO^ mutants, we found that the ectoplacental cavity failed to close in some late-lethality Trim28^KO^ embryos (Fig. 1B,D; supplementary material Fig. S2B,D) and that the allantois was always underdeveloped (supplementary material Fig. S2B,D,F,H). However, late-lethality Trim28^KO^ never showed an expansion of the chorionic trophoderm similar to Zfp568^KO^ mutants, as illustrated by the lack of the enlarged smooth yolk sac area characteristic of Zfp568^KO^ embryos (Fig. 1E,F, bracket with asterisk) (Shibata and Garcia-Garcia, 2011). Instead, the yolk sac of Trim28^KO^ mutants was covered with blisters and had a constricted appearance (Fig. 1C,D, arrowheads). Inspection of sagittal sections showed that all late-lethality Trim28^KO^ mutants had a reduced exocoelomic cavity compared with wild-type littermates (supplementary material Fig. S2), a phenotype that is likely to have contributed to their distinct constricted and collapsed appearance compared with Zfp568^KO^ embryos (Shibata and Garcia-Garcia, 2011).

Taken together, results from our phenotypic characterization suggest that Trim28 is required to control the same morphogenetic processes as Zfp568 in embryonic tissues. However, the differences between the phenotype of late-lethality Trim28^KO^ and Zfp568^KO^ embryos in extra-embryonic tissues argue that chatwo disrupts morphogenetic processes in addition to those regulated by Zfp568.

**Fig. 2.** chatwo disrupts Trim28. (A) chatwo was mapped to a 120 kb interval containing nine genes. The number of independent recombinants separating the mutation from the corresponding polymorphic markers on mouse chromosome 7 is indicated to the left. (B) Chromatograms showing Trim28 cDNA sequence in wild type and chatwo mutants. Nucleotide positions 2142-2143 of Trim28 were mutated in chatwo (highlighted in yellow) in mouse, human and Xenopus Trim28, as well as in other mouse TIF1 family members. (E-H) Whole-mount in situ hybridizations on wild-type (E), Trim28^KO^ (F) and Trim28^KO chatwo^KO^ (G,H) embryos using a probe for brachury. Scale bar: 100 μm.
Conditional inactivation of Trim28 in embryonic-derived tissues causes chatwo phenotypes

Our previous analysis of tetraploid chimeras showed that Zfp568 is required in embryonic-derived tissues to control morphogenesis of embryonic and extra-embryonic tissues (Shibata and Garcia-Garcia, 2011). If Trim28 is required to mediate Zfp568 function, we predicted that Trim28 should be required in the same tissues as Zfp568. We therefore used a floxed conditional allele of Trim28 (Trim28L2; Cammas et al., 2000; Weber et al., 2002) to conditionally inactivate Trim28 in embryonic-derived tissues using the Sox2Cre transgene, which mediates recombination in all embryonic cell types, as well as extra-embryonic mesoderm, from early developmental stages (Hayashi et al., 2002).

We found that Sox2Cre; Trim28L2/KO embryos escaped the early E5.5 lethality caused by complete loss of Trim28 function, and arrested at ~E8.5. The embryonic phenotype of Sox2Cre; Trim28L2/KO mutants strongly resembled that of Zfp568chato and Trim28chatwo embryos, including a short anterior-posterior axis, a wavy neural tube and failure to undergo gut closure (Fig. 3A-D). Consistent with a role of Trim28 in convergent extension, analysis of Foxf1 expression showed that Sox2Cre; Trim28L2/KO embryos had a shorter and wider lateral plate mesoderm (Fig. 3A,B). Sox2Cre; Trim28L2/KO embryos did not show the yolk sac protrusions or trophoblast malformations characteristic of Trim28chatwo and Zfp568chato mutants (Fig. 3B,D, arrowheads). However, similar to Zfp568 mutant embryos (Shibata and Garcia-Garcia, 2011), yolk sac vasculogenesis was disrupted in Sox2Cre; Trim28L2/KO mutants, as visualized by retention of embryonic blood cells in blood islands (not shown) and PECAM staining (Fig. 3E-H).

During early embryogenesis, Trim28 is expressed in both embryonic and extra-embryonic tissues (supplementary material Fig. S3A) (Cammas et al., 2000). In situ hybridization experiments confirmed that Trim28 expression was effectively reduced in embryonic-derived tissues of Sox2Cre; Trim28L2,KO embryos (supplementary material Fig. S3A, B, solid arrowheads) and that, consistent with the lack of Sox2Cre expression in the trophectoderm (Hayashi et al., 2002), Trim28 expression was still expressed at high levels in this tissue (supplementary material Fig. S3A, B, open arrowheads). Western blotting indicated that although conditional inactivation of Trim28 with Sox2Cre substantially reduced the levels of Trim28, a small amount of protein could still be detected in embryonic tissues (supplementary material Fig. S3C). Thus, it is possible that either Trim28 expression in the trophectoderm or the small amount of TRIM28 protein in embryonic tissues could be responsible for the milder yolk sac phenotype of Sox2Cre; Trim28L2,KO mutants compared with Trim28chatwo embryos. Regardless, the resemblance of Sox2Cre; Trim28L2,KO embryos to Zfp568 and Trim28chatwo mutants in embryonic tissues demonstrates further that Trim28 is required for mammalian convergent extension.

TRIM28 forms transcriptional repressor complexes with ZFP568

The similarities between Trim28chatwo and Zfp568chato prompted us to examine whether TRIM28 interacts physically with ZFP568 and is required for ZFP568 transcriptional activity.

Yeast two-hybrid experiments showed that TRIM28 binds ZFP568 and that the interaction is mediated by the ZFP568 KRAB domain region (Fig. 4A). The interaction between TRIM28 and ZFP568 was also observed in mammalian cells, as revealed by co-immunoprecipitation experiments (Fig. 4B). TRIM28 has been shown to be enriched at heterochromatic puncta in the nuclei of NIH3T3 cells (Nielsen et al., 1999). We found that GFP-tagged ZFP568 colocalized with TRIM28 in the nucleus of NIH3T3 cells and was present in the same heterochromatic foci (Fig. 4C). Taken together, these results show that TRIM28 and ZFP568 form protein complexes in the nucleus, consistent with a role in regulation of gene expression.

To determine whether TRIM28 mediates ZFP568 transcriptional activity, we used mammalian luciferase reporter assays. We found that GAL4DBD-ZFP568 chimeric protein efficiently repressed expression of a 5xUAS-luciferase reporter in HEK293 cells (Fig. 5A, lane 1). This repression was enhanced in a dose-dependent fashion when increasing amounts of MYC-TRIM28 were transfected (Fig. 5A, lanes 2-4). Conversely, the ability of GAL4DBD-ZFP568 to repress transcription (Fig. 5B, lanes 1,2) was reduced when endogenous levels of TRIM28 were decreased using TRIM28 siRNAs (Fig. 5B, lanes 3,4). These experiments demonstrate that ZFP568 functions as a transcriptional repressor in vitro and that its ability to repress in these luciferase assays is dependent on the level of TRIM28.
chatwo mutations affect the protein stability and repressive activity of TRIM28

To determine the molecular basis for the effects of the hypomorphic chatwo mutation on TRIM28, we first tested the ability of TRIM28<sub>chatwo</sub> to provide ZFP568-mediated transcriptional repression in luciferase assays similar to those shown in Fig. 5. We found that GAL4DBD-ZFP568 repression increased in the presence of increasing amounts of FLAG-TRIM28<sub>chatwo</sub> (Fig. 6A; compare red and gray luciferase levels), indicating that TRIM28<sub>chatwo</sub> could bind to the KRAB domain of ZFP568 and mediate transcriptional repression. In agreement with this result, we found that TRIM28<sub>chatwo</sub> was still able to interact with ZFP568 in yeast two-hybrid assays (supplementary material Fig. S4A), our results suggest that chatwo mutations affect the stability of TRIM28-KRAB domain proteins complexes, a hypothesis consistent with previous reports indicating that the stability of KRAB domain proteins depends on TRIM28 (Peng et al., 2000; Wolf and Goff, 2009).

It is noteworthy that the amount of FLAG-TRIM28<sub>chatwo</sub> protein in cells was lower than in cells transfected with an equal amount of wild-type FLAG-TRIM28 (Fig. 6A, anti-FLAG western lanes 2-7). This result was not an artifact of the tagged forms of TRIM28 used in these experiments, as we obtained similar results with different FLAG- and MYC-tagged versions of TRIM28<sub>chatwo</sub> (Fig. 6; data not shown). Moreover, the reduced protein levels produced by TRIM28<sub>chatwo</sub> transgenes appeared to be specific to the chatwo mutations, as the sumoylation-deficient FLAG-TRIM28<sub>6KR</sub> was expressed at similar levels to wild-type FLAG-TRIM28 (Fig. 6A, compare anti-FLAG western blot lanes 2-4 with lanes 8-10). Plasmids encoding FLAG-TRIM28 and FLAG-TRIM28<sub>chatwo</sub> produced similar protein levels in a reticulocyte translation system (supplementary material Fig. S5A), indicating that chatwo mutations do not affect the translation efficiency of these plasmids. Therefore, our results suggest that chatwo mutations affect the protein stability and/or rate of degradation of TRIM28.

We found that transfection of FLAG-TRIM28<sub>chatwo</sub> decreased the levels of GAL4DBD-ZFP568 protein in cells (Fig. 6A, anti-GAL4DBD western blot compare lanes 2-7 with lane 1), as well as protein levels from transgenes containing other KRAB domain proteins, including ZFP57 and ZFP809 (not shown). Because TRIM28<sub>chatwo</sub> was still able to interact with ZFP568 in yeast two-hybrid assays (supplementary material Fig. S4A), our results suggest that chatwo mutations affect the stability of TRIM28-KRAB domain proteins complexes, a hypothesis consistent with previous reports indicating that the stability of KRAB domain proteins depends on TRIM28 (Peng et al., 2000; Wolf and Goff, 2009).

Because the effects of chatwo mutations on the stability of TRIM28 and TRIM28-KRAB protein complexes could be responsible for the reduced transcriptional repression activity of FLAG-TRIM28<sub>chatwo</sub> in GAL4DBD-ZFP568 luciferase assays (Fig. 6A), we investigated further whether chatwo mutations disrupt the transcriptional repressor activity of TRIM28. For this, we tested the ability of wild-type, chatwo mutant and sumoylation-deficient versions of a GAL4DBD-TRIM28 chimeric protein to repress directly expression of the 5xUAS-luciferase reporter (Fig. 6B). As previously shown (Mascel et al., 2007), GAL4DBD-TRIM28 repressed luciferase reporter expression in a dose-dependent fashion (Fig. 6B, blue) and the sumoylation-deficient GAL4DBD-TRIM28<sub>6KR</sub> was not able to repress as efficiently (Fig. 6B, green). Similar to the effect of chatwo mutations on TRIM28 stability observed previously (Fig. 6A), cells transfected with GAL4DBD-TRIM28<sub>chatwo</sub> contained lower levels of the chimeric protein than cells transfected with the same amount of wild-type GAL4DBD-TRIM28 (Fig. 6B, compare anti-GAL4DBD western blots). However, the ability of GAL4DBD-TRIM28<sub>chatwo</sub> to repress transcription was reduced compared with similar levels of wild-type GAL4DBD-TRIM28 protein (Fig. 6B, compare 800 ng to 80 ng).
GAL4DBD-TRIM28\textsuperscript{chatwo} in lane 5 with 200 ng GAL4DBD-TRIM28 in lane 3, but was not as impaired as that of GAL4DBD-TRIM28\textsuperscript{DKR}. The ability of TRIM28\textsuperscript{chatwo} to repress transcription in the context of our cell transfection experiments is consistent with results from co-immunoprecipitation experiments showing that MYC-tagged TRIM28\textsuperscript{chatwo} could still recruit the chromatin-modifiers SETDB1 and CHD3 (supplementary material Fig. S4B). Altogether, these assays demonstrate that, independent of their effect on TRIM28 protein levels, chatwo mutations impair, but do not completely eradicate, TRIM28 repressive activity.

Repression of TRIM28 targets is disrupted in chatwo mutant embryos

To determine whether results from the molecular characterization of TRIM28’s chatwo mutations held true in vivo, we sought to determine whether the protein levels and transcriptional repressive activity of TRIM28 were disrupted in chatwo mutant embryos. Western blotting determined that E7.5 chatwo mutants contained ~40-55% the level of TRIM28 protein present in wild-type littermate embryos (Fig. 7A; supplementary material Fig. S6), but transcript levels were normal, as tested by qRT-PCR (Fig. 7B). Therefore, this result confirms our previous findings and provides evidence that chatwo mutations affect the stability and/or rate of degradation of TRIM28 during early mouse development.

TRIM28 has been reported to repress expression of retrotransposons, including IAPs (Rowe et al., 2010). Therefore, we quantified the levels of IAP expression as a readout of TRIM28\textsuperscript{chatwo} repressive activity. qRT-PCR showed that IAP elements were expressed at high levels in chatwo mutant embryos compared with wild-type littermate controls (26±3.45 fold; Fig. 7C, light gray). However, the levels of IAP expression in Trim28\textsuperscript{chatwo} mutants were not as pronounced as in Trim28\textsuperscript{KO} embryos (87.26±18.34 fold; Fig. 7C, dark gray). These experiments demonstrate that chatwo mutations disrupt, but do not completely eliminate the repressive activity of TRIM28 in vivo. Because IAP silencing was not disrupted in Zfp568\textsuperscript{chatwo} mutants, these results further corroborate that chatwo mutations affect ZFP568-independent functions of TRIM28 (Fig. 7C).

DISCUSSION

ZFP568-TRIM28 complexes control convergent extension and morphogenesis of extra-embryonic tissues

On the basis of its ability to bind KRAB domains and mediate transcriptional repression, TRIM28 has been proposed to be the universal co-repressor of all KRAB domain proteins (Urrutia, 2003). However, the roles of TRIM28 as a universal KRAB co-repressor are poorly understood, partly owing to the lack of knowledge about the biological functions of individual KRAB domain proteins. The phenotypic similarities between Trim28\textsuperscript{chatwo} and Zfp568\textsuperscript{chatwo} mutants, together with the identification of chatwo as a hypomorphic allele of Trim28, provide strong genetic evidence that ZFP568 and TRIM28 control convergent extension and morphogenesis of extra-embryonic tissues through a common molecular mechanism. This conclusion is further supported by the phenotype of embryos with a conditional inactivation of Trim28 (Fig. 3; supplementary material Table S2). We show that ZFP568 and TRIM28 interact physically and colocalize in heterochromatic foci, and demonstrate that TRIM28 is required to mediate ZFP568 transcriptional repression. These results demonstrate an essential role of TRIM28 as a co-factor of ZFP568, and are consistent with the notion that ZFP568-TRIM28 complexes control morphogenetic processes through transcriptional repression.

chatwo mutations disrupt TRIM28 protein stability and transcriptional repression activity

We have demonstrated genetically that chatwo is a hypomorphic allele of Trim28, and determined that chatwo mutations disrupt both TRIM28 protein stability and transcriptional activity in cell culture assays and in embryos.

Even though the effect of chatwo mutations on TRIM28 protein levels surely contributes to the phenotypes caused by this hypomorphic TRIM28 condition, mice with a 50% reduction in
TRIM28 levels are viable (Whitelaw et al., 2010). Therefore, we find it unlikely that the 45-60% reduction in TRIM28 protein levels caused by *chatwo* mutations, alone, can explain the lethality and severity of morphological defects in *Trim28chatwo* embryos. Instead, we favor the hypothesis that the phenotype of *Trim28chatwo* mutants originates from the effects of *chatwo* mutations on both TRIM28 protein stability and transcriptional activity.

According to the available three dimensional structure of TRIM28’s PHD-bromodomain region (Zeng et al., 2008), *chatwo* mutations are located in an area of the bromodomain facing the adjacent PHD motif. We hypothesize that *chatwo* mutations might disrupt either the folding of the bromodomain or its interaction with the adjacent PHD motif, a configuration that might be important for the ability of TRIM28 to form functional transcriptional repressor complexes. The PHD domain of TRIM28 has been shown to promote intramolecular sumoylation of the neighboring bromodomain, a modification that impacts TRIM28 transcriptional activity (Ivanov et al., 2007; Mascle et al., 2007). We find it unlikely that the effect of *chatwo* mutations on TRIM28 repression activity is due to lack of sumoylation, as *chatwo* mutations do not affect any of TRIM28’s sumoylated lysine residues, and the repressive activity of a sumoylation-deficient TRIM28 is far more reduced than that of TRIM28*chatwo* (Fig. 6B, compare TRIM28G6K*chatwo*-red and TRIM28G6K*chatwo*-green). Because TRIM28’s C-terminal bromodomain has been involved in recruitment of chromatin-modifying enzymes, including SETDB1 and CHD3 (Schultz et al., 2002; Sripathy et al., 2006), we favor the hypothesis that *chatwo* mutations affect TRIM28 repressive activity by interfering with the recruitment of these factors or other possible transcriptional co-factors that are as yet unknown.

**TRIM28 influences the stability of KRAB domain proteins**

Consistent with previous reports (Peng et al., 2000; Wolf and Goff, 2009), the molecular characterization of *chatwo* presented here indicates that the levels of TRIM28 in cells impinge on the stability of ZFP568 and other KRAB domain proteins (Fig. 6). We found that the effects of TRIM28 on KRAB domain proteins were dependent on the relative amount of each protein within the cell. Hence, increasing amounts of tagged-TRIM28 stabilized GAL4DBD-ZFP568 in a dose-dependent fashion only when the latter was transfected in excess (compare results in Fig. 6A with those of Fig. 5A). Notably, we did not observe destabilization of GAL4DBD-ZFP568 upon TRIM28 siRNA treatment (Fig. 5B), a surprising result given that stability of other KRAB domain proteins has been previously described to decrease upon TRIM28 knockdown (Wolf and Goff, 2009). We attribute this discrepancy to differences in the experimental design and/or in the affinity of distinct KRAB domain proteins for TRIM28 between our experiments and those previously published. Nevertheless, our cell culture experiments showed a consistent effect of *chatwo* mutations in destabilizing transfected GAL4DBD-ZFP568 (Fig. 6A, lanes 5-7).

Although an effect of *Trim28chatwo* mutations on ZFP568 protein stability could lead to a dominant loss-of-function effect in embryos, we have not observed any genetic evidence suggesting a possible antimorphic activity of the *chatwo* allele. Namely, we did not observe any dominant phenotype associated with *chatwo* heterozygote animals, nor did we observe a genetic interaction between *Trim28chatwo* and Zfp568*chatwo* in double heterozygote embryos (supplementary material Fig. S8). Therefore, further research is needed to investigate the molecular mechanisms underlying the effects of TRIM28 on ZFP568 and other KRAB domain proteins.
between phenotype and lethality of Trim28 chatwo mutations are sufficient to bypass TRIM28 requirements in pre-gastrula stage embryos, but not enough to fulfill the functions of TRIM28 past E7.5. Therefore, the identification and characterization of Trim28 chatwo embryos revealed that TRIM28 has separate requirements during the early stages of mouse development. Analysis of Sox2Cre; Trim28^{L2/KO} embryos also shed light on TRIM28 spatial and temporal requirements at early embryonic stages. The late lethality of Sox2Cre; Trim28^{L2/KO} embryos indicates that Trim28 might be required in embryonic tissues at early developmental stages prior to Sox2Cre activity (Hayashi et al., 2002). Alternatively, it is possible that Trim28 is required during pre-gastrula stages in the trophectoderm, a tissue that expresses high levels of TRIM28 and was still able to recognize the chatwo mutant protein.

Because the DNA target specificity of TRIM28 is thought to depend on its interaction with other factors, mainly KRAB domain proteins (Urrutia, 2003), we hypothesize that the differences between phenotype and lethality of Trim28 hypomorph (Trim28^{chatwo}), conditional (Sox2Cre; Trim28^{L2/KO}) and null (Trim28^{KO}) conditions reflect that specific co-factors require different levels of TRIM28 activity.

Additionally, we obtained data indicating that TRIM28 requirements for a particular KRAB-domain protein might differ in a tissue-specific manner. Specifically, Sox2Cre; Trim28^{L2/KO} embryos showed similar embryonic morphogenetic defects to Zfp568^{chato} and late-arrest Trim28^{chatwo} embryos, but lacked the extra-embryonic malformations characteristic of these mutants. Therefore, it is possible that ZFP568 requires higher levels of TRIM28 to control morphogenesis of embryonic tissues than to fulfill the functions of TRIM28 past E7.5.
promote the development of the yolk sac and placenta. These differential requirements might be dictated by tissue-specific factors that could stabilize or modulate the activity of TRIM28 complexes with KRAB domain proteins or with other transcription factors.

In conclusion, the phenotypic and molecular characterization of the Trim28<sup>−/−</sup> hypomorphic allele described here provides strong genetic evidence that TRIM28 is required for ZFP568 function, identifies a novel role of Trim28 in the control of mammalian convergent extension and reveals separate functions of Trim28 during early mouse embryogenesis. Recent studies to identify TRIM28 genomic targets have challenged the role of KRAB during early mouse embryogenesis. Recent studies to identify genetic evidence that TRIM28 is required for ZFP568 function, the authors declare no competing financial interests.

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The authors declare no competing financial interests.
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