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

Maho Shibata¹, Kristin E. Blauvelt¹, Karel F. Liem, Jr² and María J. García-García¹,*

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 (KRIP-1), 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 (CEBPβ – 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., 2011; Moosmann et al., 1996; Urrutia, 2003). Although KRAB domain zinc fingers represent the largest family of transcription

1Department of Molecular Biology and Genetics, Cornell University, 526 Campus Road, Ithaca, NY 14853, USA. 2Sloan-Kettering Institute, Developmental Biology Program, 1275 York Avenue, New York, NY 10065, USA.
*Author for correspondence (garciamj@cornell.edu)

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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 still limited. Furthermore, it is not yet clear whether TRIM28 is indispensable for the activity of individual KRAB domain proteins.

Our previous characterization of Zfp568chato mutants identified roles for the KRAB zinc finger protein ZFP568 in the control of convergent extension and morphogenesis of extra-embryonic tissues (Garcia-Garcia et al., 2008; Shibata and Garcia-Garcia, 2011). 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-nitrosourea (ENU)-induced mutation that causes a similar phenotype to Zfp568 mutants, is a hypomorphic allele of Trim28. Our comparative analysis of Trim28hcko 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 Mus musculus strain backgrounds. Phenotype expressivity was quantified in embryos from congeneric FvB/NJ and N7 backcrossed C57BL/6 animals. D7Mit178 and D7Mit76 polymorphic markers were used for genotyping. Zfp568hako (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; Trim282/2; KO or Trim282/2; KO females were crossed to Sox2Cre; Trim282/2; KO 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, Zfp568hako, 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-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 co-transfected with the Dual-Luciferase Reporter System (Promega) 24 hours after transfection. For small interfering RNA (siRNA) knockdown, 8 pmol of Trim28 siRNAs #1 (1979), #2 (1978) or non-silencing siRNA (Ambion) was transfected using Lipofectamine RNAiMax (Invitrogen). Cells were transfected with luciferase effectors and reporters 24 hours after siRNA transfection and luciferase was assayed after another 48 hours. 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 transcription 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 (MA1-2023, Thermo Scientific; 1:25), anti-CHD3 (Abcam; 1:700), anti-SETDB1 (Millipore; 1:1000), anti-GAL4DBD (RK5C1, Santa Cruz Biotechnology; 1:500-1:8000), anti-Myc (9e10, Hybridoma Bank; 1:250-1:10000), 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), anti-mouse/rabbit HRP (Jackson ImmunoResearch; 1:10,000), anti-PECAM (eBioscience; 1:200), anti-rat Alexa 488 (Molecular Probes; 1:200), anti-mouse Alexa 568 (Molecular Probes; 1:200).

**Constructs and primers**

Plasmids pCDNA3.1-Gal4DBD-TRIM28, pCDNA3.1-Gal4DBD-TRIM28K0, pGL35XUAS firefly luciferase and pRL Renilla luciferase are generated using primers as indicated in supplementary material Table S1. 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. 1A). **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 homozygous 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** was used 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; Moosmann 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 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 (**Trim28**KO)** 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 **Trim28chato** 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 **Trim28chato** could be a hypomorphic allele. To obtain genetic confirmation that **chatwo** disrupts **Trim28**, we analyzed **Trim28**chato/KO embryos. Like **Trim28chato** mutants, some **Trim28chato**KO 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 **Trim28chato**KO embryos examined, regardless of their stage of lethality, indicating that phenotypes of **Trim28chato**KO embryos are milder than those of **Trim28**KO mutants (Fig. 2E-H). These results support the hypothesis that **chatwo** mutations create a hypomorphic allele of **Trim28**.

**chatwo affects ZFP568-dependent and ZFP568-independent roles of TRIM28**

We showed previously that Zfp568chato 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 Zfp568cha20 embryos and Trim28cha20 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 Trim28cha20 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 Trim28cha20 embryos (not shown). Additionally, late-lethality Trim28cha20 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 Zfp568cha20 mutants (Garcia-Garcia et al., 2008). We found that in late-lethality Trim28cha20 embryos the definitive endoderm (Ttr-negative) failed to narrow (supplementary material Fig. S2E,G) to the same extent as Zfp568cha20 embryos (Garcia-Garcia et al., 2008). Altogether, our analysis shows that the embryonic defects in late-lethality Trim28cha20 embryos strongly resemble defects in Zfp568cha20 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 Zfp568cha20 mutants and late-lethality Trim28cha20 embryos, although some phenotypic differences were notable. Like Zfp568cha20 embryos, the yolk sac of Trim28cha20 mutants had numerous bubble-like protrusions (Fig. 1C,D, arrowheads). However, yolk sac blisters in late-lethality Trim28cha20 embryos were found throughout the entire yolk sac, whereas they often clustered in a region proximal to the embryo in Zfp568cha20 mutants (Fig. 1C-F) (Shibata and Garcia-Garcia, 2011). In Zfp568cha20 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 Zfp568cha20 mutants, we found that the ectoplacental cavity failed to close in some late-lethality Trim28cha20 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 Trim28cha20 never showed an expansion of the chorionic trophoderm similar to Zfp568cha20 mutants, as illustrated by the lack of the enlarged smooth yolk sac area characteristic of Zfp568cha20 embryos (Fig. 1E,F, bracket with asterisk) (Shibata and Garcia-Garcia, 2011). Instead, the yolk sac of Trim28cha20 mutants was covered with blisters and had a constricted appearance (Fig. 1C,D, arrowheads). Inspection of sagittal sections showed that all late-lethality Trim28cha20 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 Zfp568cha20 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 Trim28cha20 and Zfp568cha20 embryos in extra-embryonic tissues argue that cha20 disrupts morphogenetic processes in addition to those regulated by Zfp568.
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 (Trim28<sup>L2</sup>; 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; Trim28<sup>L2/KO</sup> embryos escaped the early E.5.5 lethality caused by complete loss of Trim28 function, and arrested at ~E8.5. The embryonic phenotype of Sox2Cre; Trim28<sup>L2/KO</sup> mutants strongly resembled that of Zfp568<sup>chatwo</sup> and Trim28<sup>chatwo</sup> embryos, including a shorter 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; Trim28<sup>L2/KO</sup> embryos had a shorter and wider lateral plate mesoderm (Fig. 3A,B). Sox2Cre; Trim28<sup>L2/KO</sup> embryos did not show the yolk sac protrusions or trophoblast malformations characteristic of Trim28<sup>chatwo</sup> and Zfp568<sup>chatwo</sup> mutants (Fig. 3B,D, arrowheads). However, similar to Zfp568 mutant embryos (Shibata and Garcia-Garcia, 2011), yolk sac vasculogenesis was disrupted in Sox2Cre; Trim28<sup>L2/KO</sup> 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; Trim28<sup>L2/KO</sup> 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; Trim28<sup>L2/KO</sup> mutants compared with Trim28<sup>chatwo</sup> embryos. Regardless of the resemblance of Sox2Cre; Trim28<sup>L2/KO</sup> embryos to Zfp568 and Trim28<sup>chatwo</sup> mutants in embryonic tissues demonstrates further that Trim28 is required for mammalian convergent extension.

TRIM28 forms transcriptional repressor complexes with ZFP568

The similarities between Trim28<sup>chatwo</sup> and Zfp568<sup>chatwo</sup> 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 (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 nuclei of NIH3T3 cells and was present in the same heterochromatin 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 a 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.

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**Fig. 3.** Trim28 is required in embryonic-derived cells. (A-H) Wild-type (A,C,E,G) and Sox2Cre; Trim28<sup>L2/KO</sup> (B,D,F,H) E8.5 mouse embryos were analyzed by whole-mount in situ hybridization with a Foxf1 probe (A,B) and in sagittal sections (C,D). Yolk sacs were stained with anti-PECAM antibodies to highlight the vascular plexus (E-H). Brackets in A and B highlight the lateral plate mesoderm. Arrowheads point to the yolk sac, which has a smooth appearance in Sox2Cre; Trim28<sup>L2/KO</sup> embryos. TE, trophectoderm; al, allantois. G and H are high magnifications of E and F, respectively. Scale bars: 100 μm.
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 TRIM28chatwo 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-TRIM28chatwo (Fig. 6A; compare red and gray luciferase levels), indicating that TRIM28chatwo could bind to the KRAB domain of ZFP568 and mediate transcriptional repression. In agreement with this result, we found that TRIM28chatwo was still able to interact with ZFP568 in yeast two-hybrid assays (supplementary material Fig. S4A). GAL4DBD-ZFP568 transcriptional repression in the presence of ectopic FLAG-TRIM28chatwo was slightly reduced compared with when similar amounts of wild-type FLAG-TRIM28 were transfected (Fig. 6A, compare blue and red bars), but was not as severely affected compared with similar experiments using a sumoylation-deficient FLAG-TRIM28KR (previously described to impair TRIM28 repressive activity) (Mascle et al., 2007) (Fig. 6A, compare red and green bars). These results indicate that chatwo mutations decrease, but do not completely disrupt, TRIM28-mediated ZFP568 transcriptional repression activity.

It is noteworthy that the amount of FLAG-TRIM28chatwo 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 TRIM28chatwo (Fig. 6; data not shown). Moreover, the reduced protein levels produced by TRIM28chatwo transgenes appeared to be specific to the chatwo mutations, as the sumoylation-deficient FLAG-TRIM286KR 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-TRIM28chatwo 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-TRIM28chatwo 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 TRIM28chatwo 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-TRIM28chatwo 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 (Mascle et al., 2007), GAL4DBD-TRIM28 repressed luciferase reporter expression in a dose-dependent fashion (Fig. 6B, blue) and the sumoylation-deficient GAL4DBD-TRIM286KR 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-TRIM28chatwo 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-TRIM28chatwo to repress transcription was reduced compared with similar levels of wild-type GAL4DBD-TRIM28 protein (Fig. 6B, compare 800 ng

Fig. 4. ZFP568 interacts with TRIM28. (A) Yeast two-hybrid assays showed interaction of GAL4DBD-ZFP568 (full length and KRAB domains constructs) with GAL4AD-TRIM28, as indicated by growth in Ade-His-Leu-Trp-media (top panel) and blue colony color in Leu-Trp-X-alpha-gal plates (lower panel). GAL4DBD-ZFP568 zinc finger region did not interact with GAL4AD-TRIM28. Lamin C (LmnC) was used as a negative control. p53 interaction with SV40 large T-antigen was used as a positive control. (B) HA-ZFP568 and MYC-TRIM28 co-immunoprecipitate (IP) when transfected in HEK293 cells. (C) Subcellular localization of TRIM28 (red) and GFP-ZFP568 (top row, green) in NIH3T3 cells. An empty GFP vector was used as control (bottom panels, green). Samples were co-stained with DAPI. Scale bar: 50 μm.
Western blotting determined that E7.5 activity of TRIM28 were disrupted in determine whether the protein levels and transcriptional repressive
mutant embryos
Repression of TRIM28 targets is disrupted in effect on TRIM28 protein levels, Altogether, these assays demonstrate that, independent of their modifiers SETDB1 and CHD3 (supplementary material Fig. S4B).

TRIM28's mutations held true in vivo, we sought to determine whether the protein levels and transcriptional repression 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.

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 corepressor are poorly understood, partly owing to the lack of knowledge about the biological functions of individual KRAB domain proteins. The phenotypic similarities between Trim28chatwo and Zfp568chato 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, which display defects similar to Trim28chatwo and Zfp568chato mutants in embryonic tissues (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 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 levels 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 transected 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 antimeromic 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 Zfp568chatwo in double heterozygote embryos (supplementary material Fig. S8). Therefore, further
between This is consistent with results from the double mutant analysis roles of TRIM28 in the development of the yolk sac and placenta. 1; supplementary material Table S2) uncovered ZFP568-independent similar to those of Zfp568chato Trim28chatwo embryonic phenotypes of between ZFP568 and TRIM28, the differences between the extra- chatwo mutations disrupt ZFP568-independent KRAB domain proteins in vivo. KRAB domain proteins will be required to address whether the requirements for a particular KRAB-domain protein might differ (Urrutia, 2003), we hypothesize that the differences requirements at early embryonic stages. The late lethality of chatwo caused by TRIM28 antibody and lysates from a gradient of decreasing amounts of E7.5 wild-type extract (lanes 1-5) and a pool of six chatwo mouse embryos (lane 6). Lanes 1 to 5 represent lysates from approximately 4, 2, 1, 0.5 and 0.25 wild-type embryos, respectively. Anti-GAPDH antibody was used as a loading control (lower panel). The TRIM28 antibody was raised against part of the coiled-coil and HP1-binding domain and was still able to recognize the chatwo mutant protein. Similar results were obtained using an anti-TRIM28 polyclonal antibody (data not shown). Quantification of TRIM28 levels is shown in supplementary material Fig. S6. Note that mRNA and protein levels of Trim28 were unaffected in Zfp568chato mutants and that Zfp568 expression was normal in Trim28chatwo embryos (supplementary material Fig. S7). B) qRT-PCR analysis of Trim28 expression in wild-type and Trim28chatwo embryos. C) Expression of IAP elements quantified by qRT-PCR in wild-type, Zfp568chato, Trim28chatwo and Trim28KO embryos. qRT-PCR results show expression relative to the wild-type sample and normalized with respect to expression of Gapdh. Error bars represent s.d. between independent pools of embryos.

experiments using antibodies against ZFP568 and other specific KRAB domain proteins will be required to address whether the levels of TRIM28 within cells might be important to stabilize KRAB domain proteins in vivo.

chatwo mutations disrupt ZFP568-independent functions of TRIM28

Whereas the phenotypic similarities between Trim28chatwo and Zfp568chato mutants in embryonic tissues revealed a functional link between ZFP568 and TRIM28, the differences between the extra-embryonic phenotypes of Trim28chatwo and Zfp568chato embryos (Fig. 1; supplementary material Table S2) uncovered ZFP568-independent roles of TRIM28 in the development of the yolk sac and placenta. This is consistent with results from the double mutant analysis between Trim28chatwo and Zfp568chato mutants (supplementary material Fig. S8), which showed that embryos simultaneously mutant for Trim28chatwo and Zfp568chato have extra-embryonic defects more similar to those of Trim28chatwo embryos. Additionally, we have obtained evidence indicating that chatwo mutations affect ZFP568-independent functions of TRIM28 that relate to silencing of retroviral elements and gastrulation processes required for embryo survival past E7.5. We speculate that these ZFP568-independent roles of TRIM28 could be carried out through its ability to interact with other KRAB domain proteins or additional proteins that might determine its transcriptional activity and/or targets.

TRIM28 is differentially required during early mouse embryogenesis

Embryos homozygous for the Trim28chatwo hypomorph condition survive longer than Trim28KO mice, which fail to gastrulate and which die at E5.5 (Cammas et al., 2000). This observation implies that the reduced TRIM28 protein levels and repressive activity caused by 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 Trim28chatwo embryos revealed that TRIM28 has separate requirements during the early stages of mouse development. Analysis of Sox2Cre; Trim28−/−embryos also shed light on TRIM28 spatial and temporal requirements at early embryonic stages. The late lethality of Sox2Cre; Trim28−/−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 is not affected in Sox2Cre; Trim28−/−embryos (supplementary material Fig. S3).

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 hypomorphic (Trim28−/−), conditional (Sox2Cre; Trim28−/−) and null (Trim28−/−) 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−/−embryos showed similar embryonic morphogenetic defects to Zfp568chato and late-arrest Trim28chatwo 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
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 \textit{Trim28}\textsuperscript{HetD} hypomorphic allele described here provides strong genetic evidence that TRIM28 is required for ZFP568 function, identifies a novel role of \textit{Trim28} in the control of mammalian convergent extension and reveals separate functions of \textit{Trim28} during early mouse embryogenesis. Recent studies to identify TRIM28 genomic targets have challenged the role of KRAB domain proteins as determining factors for the biological functions TRIM28 genomic targets have challenged the role of KRAB during early mouse embryogenesis. Recent studies to identify convergent extension and reveals separate functions of identifies a novel role of

Supplementary material

The authors declare no competing financial interests.

Competing interests statement

This work was supported by the National Institutes of Health [grant number R01HD060581 from the Eunice Kennedy Shriver National Institute of Child Health & Human Development to M.J.G., R01NS04385 to K.F.L.]; and by the National Science Foundation [IOS-1020878 to M.J.G.G.]. Deposited in PMC for release after 12 months.

References


TRIM28 is required by ZFP568

Utilization of a whole genome SNP panel for efficient genetic mapping in the mouse. Genome Res. 16, 436-440.


Table S1. Primers

<table>
<thead>
<tr>
<th>Vector/Plasmid</th>
<th>Restriction Enzyme</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td><em>chatwo</em>-RFLP with <em>Bst</em>ll</td>
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<td>IAP 5’UTR for qRT-PCR</td>
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### Table S2. Summary of the comparative phenotypic analysis of Zfp568\textsuperscript{chato}, Trim28\textsuperscript{chatwo}, Trim28\textsuperscript{chatwo/KO}, Trim28\textsuperscript{KO/KO} and Sox2Cre; Trim28\textsuperscript{L2/KO} embryos

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Embryonic arrest at</th>
<th>brachyury expression</th>
<th>Convergent extension defects</th>
<th>Yolk sac blistering</th>
<th>Yolk sac vasculogenesis defects</th>
<th>Allantois defects</th>
<th>Expanded chorion</th>
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<td>Zfp568\textsuperscript{chato}</td>
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<td>normal\textsuperscript{1}</td>
<td>yes (Fig. 1)\textsuperscript{1}</td>
<td>yes\textsuperscript{1}</td>
<td>yes\textsuperscript{1}</td>
<td>yes\textsuperscript{1}</td>
<td>yes (Fig. 1)\textsuperscript{1}</td>
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<tr>
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<td>E8.5</td>
<td>normal (Fig. S1)</td>
<td>yes (Fig. 1)</td>
<td>yes (Figs 1, S1, S2)</td>
<td>yes (ns)</td>
<td>yes (Fig. S2)</td>
<td>no (Figs 1, S2)</td>
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<tr>
<td></td>
<td>E7.5</td>
<td>normal (Fig. S1)</td>
<td>*</td>
<td>yes (Fig. S1)</td>
<td>*</td>
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<td>*</td>
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<tr>
<td>Trim28\textsuperscript{chatwo/KO}</td>
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<td>normal (Fig. 2)</td>
<td>yes (Fig. 2; ns)</td>
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<td>na</td>
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<tr>
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<td>normal (Fig. 2)</td>
<td>*</td>
<td>yes (Fig. 2)</td>
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<td>*</td>
<td>*</td>
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<td>absent (Fig. 2)\textsuperscript{8}</td>
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<td>yes\textsuperscript{1}</td>
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<td>Sox2Cre; Trim28\textsuperscript{L2/KO}</td>
<td>&gt;E8.5</td>
<td>normal (ns)</td>
<td>yes (Fig. 3)</td>
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<td>yes (Fig. 3)</td>
<td>yes (Fig. 3)</td>
<td>no (Fig. 3)\textsuperscript{**}</td>
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</table>

The figures or previous publications providing evidence for each of the phenotypic features are indicated in brackets or superscripts, respectively. Boxes shaded in light gray indicate the most common phenotype to all mutant conditions analyzed. Dark gray-shaded boxes indicate phenotypic features that, for some mutants, differed from those most commonly observed in the other conditions analyzed.

\textsuperscript{1}Garcia-Garcia et al., 2008.

\textsuperscript{8}Shibata and Garcia-Garcia, 2011.

\textsuperscript{7}Cammas et al., 2000.

ns, results not shown; na, not assayed.

*Features for which analysis is prevented by the early lethality of the mutant embryos.

**Phenotypes could be due to the presence of wild-type TRIM28 in Sox2Cre; Trim28\textsuperscript{L2/KO} embryos as shown in supplementary material Fig. S3 and mentioned in the Results section.