RESEARCH ARTICLE

Tbx6 controls left-right asymmetry through regulation of Gdf1

Daniel Concepcion1, Hiroshi Hamada2 and Virginia E. Papaioannou1,*

ABSTRACT

The Tbx6 transcription factor plays multiple roles during gastrulation, somite formation and body axis determination. One of the notable features of the Tbx6 homozygous mutant phenotype is randomization of left/right axis determination. Cilia of the node are morphologically abnormal, leading to the hypothesis that disrupted nodal flow is the cause of the laterality defect. However, Tbx6 is expressed around but not in the node, leading to uncertainty as to the mechanism of this effect. In this study, we have examined the molecular characteristics of the node and the genetic cascade determining left/right axis determination. We found evidence that a leftward nodal flow is generated in Tbx6 homozygous mutants despite the cilia defect, establishing the initial asymmetric gene expression in Dand5 around the node, but that the transduction of the signal from the node to the left lateral plate mesoderm is disrupted due to lack of expression of the Nodal coligand Gdf1 around the node. Gdf1 was shown to be a downstream target of Tbx6 and a Gdf1 transgene partially rescues the laterality defect.

KEY WORDS: Tbx6, Gdf1, Left-right asymmetry, Mouse development, Axis determination

INTRODUCTION

The bilateral symmetry of the early mouse embryo is broken during early gastrulation when genes are asymmetrically expressed around the embryonic node and in the lateral plate mesoderm (LPM). The earliest known morphological left-right asymmetry in the mouse is the leftward displacement of the future atrioventricular canal, followed by the dextral looping of the linear heart tube. As development proceeds, the embryo undergoes a process of axial rotation towards its right side and additional morphological asymmetries arise in virtually all internal organ systems (Beddington and Robertson, 1999; Brown and Anderson, 1999; Levin et al., 1995; Miller and White, 1998). The initial asymmetric gene expression around the node is driven by the Nodal coligand Gdf1 around the node. This study focuses on the T-box gene Tbx6 that is expressed between embryonic day (E) 6.5 and E13.5 in the primitive streak, the presomitic mesoderm and the tail bud, but not in the node, although node precursors do express the gene (Concepcion et al., 2017). Tbx6 null mutants die around E9.5 with defects in anterior somite patterning, ectopic neural tubes and an enlarged tail bud (Chapman et al., 1996; Chapman and Papaioannou, 1998; Takemoto et al., 2011). They display heterotaxia with randomized direction of embryo turning and heart looping and have defects in node and cilia morphology. Dll1 and Nodal expression is reduced in the perinodal region and most mutant embryos show an absence of expression of Nodal, Pitx2 and Lefty2 in the LPM as well as a lack of Ca2+ signaling at the node (Hadjantonakis et al., 2008). The mechanism by which Tbx6 affects the development of the node and node cilia and how this affects the expression of left-right specific genes in the LPM is not fully understood. In this study we found that despite the severe defect in nodal cilia, there appears to be a functional leftward nodal flow and the main effect of Tbx6 on left-right axis determination is downstream of nodal flow and the detection of the flow by perinodal crown cells. Furthermore, we identified Gdf1 as a downstream target of Tbx6 in the transduction of the perinodal Nodal signal to the LPM.

RESULTS

Node development in Tbx6 homozygous mutants

In trying to understand how Tbx6 affects the development of the nodal cilia, we examined the expression of several transcription factor genes known to affect node or cilia development and left-right axis determination. Noto is expressed at the anterior end of the primitive streak in precursors of the embryonic node and later in the node and developing notochord (Fig. 1A-D) (Beckers et al., 2007). Tbx6 homozygotes have no detectable Noto expression at the midstreak stage (Fig. 1E); at late streak, allantoic bud and head-fold stages, half of the mutants had weak, punctate expression in the LPM as well as a lack of Ca2+ signaling at the node (Hadjantonakis et al., 2008). The mechanism by which Tbx6 affects the development of the node and node cilia and how this affects the expression of left-right specific genes in the LPM is not fully understood. In this study we found that despite the severe defect in nodal cilia, there appears to be a functional leftward nodal flow and the main effect of Tbx6 on left-right axis determination is downstream of nodal flow and the detection of the flow by perinodal crown cells. Furthermore, we identified Gdf1 as a downstream target of Tbx6 in the transduction of the perinodal Nodal signal to the LPM.

In contrast, mutant (n=16) and wild-type embryos showed similar Foxf1 expression in precursors of the node at the streak stage and in the node through early somite stages (Fig. 1I-L and not shown) (Brody et al., 2000), with the exception of a single mutant with an irregular pattern at the early bud stage. Rfx3 expression in the...
Embryonic node between 0 bud and head-fold stages was indistinguishable between wild type and mutants (n=15) (Fig. 1M-P) (Bonnafe et al., 2004).

Several lines of evidence implicate non-canonical Wnt signaling in the determination of left-right asymmetry and node development in vertebrates (Kitajima et al., 2013; Minegishi et al., 2017; Oishi et al., 2006; Park et al., 2006; Zhang and Levin, 2009). We investigated the expression of the non-canonical Wnt signaling ligands 
\textit{Wnt5a} and \textit{Wnt5b}, and the receptors \textit{Fzd2}, \textit{Fzd3} and \textit{Fzd10}, all of which overlap to some extent with \textit{Tbx6} expression from primitive streak to early somite stages. \textit{Tbx6} homozygous mutant embryos showed normal expression of all of these components of the non-canonical Wnt signaling pathway (Fig. S1).

Is there functional nodal flow in \textit{Tbx6} mutant embryos?

In our previous study, we observed reduced \textit{Nodal} expression around the node but did not draw conclusions about asymmetry of expression due to the low level of expression observed (Hadjantonakis et al., 2008). Thus it remained an open question whether an asymmetric signal was present at the node. As an alternative readout of nodal flow, we examined the perinodal expression of \textit{Dand5} and found that expression appeared to be greater on the right side of the node in 7/8 mutants, with undetectable expression in the eighth embryo (Fig. 2A). Although the number of embryos is small, this was the first indication that a directional nodal flow is detected by the crown cells in spite of abnormal cilia and the apparent absence of perinodal intracellular Ca\textsuperscript{2+} signaling in \textit{Tbx6} mutant embryos (Hadjantonakis et al., 2008).

To investigate this phenomenon further and to ascertain whether the asymmetric perinodal signal was mediated through the crown cell cilia in the established pathway, we made use of a null mutation in \textit{Pkd2}, a calcium permeable ion channel gene shown to be necessary for the perinodal cilia to sense the nodal flow and initiate asymmetric gene expression (Pennekamp et al., 2002; Yoshiba et al., 2012). We produced compound \textit{Tbx6}; \textit{Pkd2} mutants and assessed embryos for expression of \textit{Pitx2} in the LPM of 2-7 somite-stage embryos or inflow tract (IFT) of the heart of more advanced embryos. The majority of \textit{Tbx6} mutants (18/26) had no \textit{Pitx2} expression in the LPM/IFT, whereas the remainder (8/26) had left-sided expression (Fig. 2B; Table S1). Most \textit{Pkd2} mutants had no or bilateral expression (18/28 and 7/28, respectively), while two had
Fig. 2. Asymmetric gene expression in Tbx6 mutant embryos, lack of perinodal Gdf1 expression and rescue by Gdf1 transgene. (A) Perinodal expression of Dand5 in wild-type and Tbx6 homozygous mutant embryos. Expression is more intense on the right side of the node in at least four out of seven wild-type embryos and in all but one of the mutant embryos (far right) (P>0.05; Fisher’s exact test). An additional mutant embryo had no visible expression and is not shown. All panels are ventral views with anterior to the bottom of the panel. L, left side; R, right side. (B) Graphical representation of the proportion of embryos expressing Pitx2 in the LPM/IFT of embryos of the indicated genotypes, with (+TG) or without the Gdf1 node-Tg. Numbers in the bars are the n values for each category. (C) Expression of Gdf1 in wild-type (A-H) and Tbx6 homozygous mutant embryos (I-P) between late head-fold (LHF) and seven somite stages. Left lateral (A-D,I-L) and ventral (E-H,M-P; anterior at the bottom) views showing Gdf1 expression in the perinodal region of wild-type embryos at LHF stage and bilaterally in the lateral plate mesoderm and perinodal region at somite stages, whereas mutant embryos lack perinodal expression at all stages (n=12). Arrows point to expression in the perinodal region; arrowheads point to expression in the LPM. a, allantois; h, heart; hf, head folds.

left-sided and one had right-sided expression (see Fig. S2). The majority of double-homozygous Tbx6; Pkd2 mutants lacked Pitx2 expression (8/10) with one embryo showing left-sided and one showing bilateral expression.

Additional Tbx6; Pkd2 double homozygotes from this same breeding cohort that also carried a transgene for Gdf1 (+TG in Fig. 2B; see later section; Table S1) had no (8/9) or right-sided (1/9) expression. Taken together, the double homozygotes do not show a bias toward left-sided expression (1/19), as do the Tbx6 homozygous mutants that show sporadic Pitx2 expression (8/8), leading to the conclusion that the effect of Tbx6 on left-biased expression of Pitx2 is hypostatic to the effect of Pkd2, as would be expected on the basis of the generally accepted pathway for detection of the nodal flow. The fact that sporadic expression of Pitx2 in Tbx6 mutants is left-sided rather than random further supports the conclusion from Dand5 expression that there is a functional leftward nodal flow.

Tbx6 is upstream of Gdf1

With evidence for an asymmetric signal at the node of Tbx6 mutant embryos mediated through detection of a nodal flow by perinodal cilia, we investigated the next step in the cascade by examining expression of Gdf1, which is essential for transfer of the perinodal Nodal signal to the left LPM. At the LHF stage, wild-type embryos express Gdf1 throughout the embryo with discrete bilateral expression in the perinodal region. Between zero and seven somite stages, Gdf1 is expressed bilaterally in both the perinodal region and LPM (Fig. 2C: B-D,F-H) (Rankin et al., 2000). In Tbx6 mutant embryos, Gdf1 is expressed uniformly throughout the embryo at the LHF stage (n=2) and bilaterally in the LPM at later stages (n=10); however, no signal is observed in the perinodal region at any stage (Fig. 2C: I-P), leading to the hypothesis that Tbx6 regulates Gdf1 in the perinodal region.

Using Conservation-Aided Transcription-Factor-Binding Site Finder (COTRASIF; http://biomed.org.ua/COTRASIF/) with a position weight matrix for Tbx6 to scan 2 kb upstream and downstream of the transcriptional start site of Gdf1, we found five putative Tbx6 binding sites (Fig. 3A; Table S2). Electrophoretic mobility shift assays (EMSA) were used to test whether Tbx6 protein binds to fluorescently labeled DNA probes for each of the five sites in vitro. Probes with mutated sites (Table S2) were used to test binding specificity. Results show that Tbx6 protein can bind specifically to sites #1, 4, and 5. Site #3 showed non-binding and site #2 showed non-specific binding in which both the wild-type and mutated probe bound to Tbx6 protein (Fig. 3B). Luciferase assays were used to test whether Tbx6 can regulate gene expression and whether it does so through the putative binding sites in the Gdf1 promoter. NIH 3T3 cells were transfected with a Tbx6 expression construct and a luciferase reporter containing 2.3 kb of the Gdf1 promoter region or the Gdf1 promoter region with mutated putative Tbx6 binding sites (excluding site #5) (Fig. 3C). Transfection of increasing amount of Tbx6 expression construct leads to a twofold increase in luciferase reporter activity. However, no difference in activity between wild-type and mutant luciferase reporters was observed (Fig. 3D). Western blot demonstrates that transfected cells express
Tbx6 protein (Fig. 3E). Thus while Tbx6 regulates luciferase activity from the Gdf1 promoter, it does not do so through these putative binding sites.

Embryos homozygous mutant for either Tbx6 or Gdf1 have randomization of heart looping and embryo turning, while heterozygous embryos have normal situs (Hadjantonakis et al., 2008; Rankin et al., 2000). To determine the extent of interaction between Tbx6 and Gdf1 we crossed Tbx6 and Gdf1 heterozygous mice to produce double heterozygotes. Normal direction of heart looping and embryo turning was observed in all Tbx6+/−;Gdf1+/- double heterozygous embryos (n=21). For a more sensitized screen, the dose of Tbx6 was further reduced using a hypomorphic allele, Tbx6rv, (Watabe-Rudolph et al., 2002) in combination with the null allele. Tbx6−/−;Gdf1−/− mutant embryos also had normal situs at E9.5 (n=11).

**Gdf1 perinodal expression in Tbx6−/− mutants rescues asymmetric expression of Pitx2**

We made use of a transgene, node-Tg, that expresses the cDNA of Gdf1 bilaterally in the perinodal region to restore Gdf1 expression in Tbx6 homozygous mutants. In the first series of experiments crossing the Tbx6 mutant stock with mice carrying the Gdf1 transgene, all wild-type embryos at E8.5 showed expression of Pitx2 in the left IFT of the heart (n=13), whereas the majority of Tbx6−/−;node-Tg transgenic embryos showed Pitx2 expression only in the right IFT (n=11).

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homozygous mutants had no detectable Pitx2 expression in the IFT (n=26/27). In Tbx6+/−;node-Tg embryos, Pitx2 expression was restored in the IFT in 8/22 (36%) embryos, all on the left side (Fig. 4).

The node-Tg transgene was also included in the previously described Tbx6; Pkd2 cross (Fig. 2B; Table S1) where the proportion of Tbx6 mutant embryos expressing left-sided Pitx2 was 31% (8/26), which was further increased to 44% (17/39) in the presence of the transgene. Combining data from both series of Gdf1 rescue experiments, 25/61 (41%) of Tbx6 homozygous mutants with Gdf1 restored in the perinodal region showed left-sided Pitx2 expression (with a single embryo showing right-sided expression), compared with 8/53 (15%) showing left-sided expression without the transgene (X^2=8, P<0.01). This indicates that perinodal Gdf1 can partially rescue asymmetric Pitx2 expression in Tbx6 mutant embryos and furthermore provides additional evidence for a functional leftward nodal flow in Tbx6 mutant embryos as the rescued embryos showed predominantly left-sided Pitx2 expression. As expected, the transgene had no apparent effect on the Pkd2 phenotype.

**DISCUSSION**

**Node formation and ciliogenesis**

We investigated genes known to be involved in node and cilia development and found that expression of the homeobox gene Noto, a gene affecting node structure and cilia motility (Beckers et al., 2007), was delayed or irregular in half of the Tbx6 mutants. However, the significance for this expression change in node and cilia formation is not clear as the expression of the downstream genes that mediate the effect on cilia, FoxJ1 and Rfx3 (Alten et al., 2012; Bonnafé et al., 2004; Brody et al., 2000) is unaffected in mutants. Since Noto is a marker for the node, the irregular expression pattern observed may be simply a reflection of node morphological irregularities previously observed in Tbx6 homozygous mutants (Hadjantonakis et al., 2008).

Previous work in Xenopus, zebrafish and mice implicated the non-canonical Wnt pathway in node and cilia development and left-right axis formation (Etheridge et al., 2008; Hamblet et al., 2002; Hashimoto et al., 2010; Kitajima et al., 2013; Minegishi et al., 2017; Oishi et al., 2006; Zhang and Levin, 2009). None of the non-canonical ligands or receptors we examined, however, differed in expression between mutants and controls.

**Asymmetric gene expression in the perinodal region and LPM**

In our previous study, we postulated that the nodal flow was disrupted by the altered motility of the abnormal cilia in Tbx6 mutants, accounting for a lack of Ca^{2+} signaling at the periphery of the node and subsequent disruption of left-right axis determination (Hadjantonakis et al., 2008). There was no expression of asymmetric genes in the LPM although we detected Nodal expression perinodally at a level too low to determine whether it was asymmetric. However, work by others has shown that a low level of perinodal Nodal expression is sufficient to initiate expression of left- (or right-) specific genes in the LPM (Brennan et al., 2002). Thus in the present study, we used an additional marker of asymmetry to determine whether the perinodal signal was asymmetric or random in Tbx6 mutant embryos. We detected asymmetric right-sided expression of Dand5, a Nodal antagonist that is responsible for the robust asymmetric expression of Nodal and is the initiator of the asymmetric molecular cascade (Babu and Roy, 2013). Although the number of embryos examined was small, two additional lines of evidence support this conclusion. We found a proportion of Tbx6 mutant embryos with sporadic Pitx2 expression, which was on the left side in 8/8 embryos. Removal of Pkd2, a gene central to the detection of the nodal flow (Pennekamp et al., 2002; Yoshida et al., 2012), from Tbx6 mutants removed this left-bias, indicating that the nodal flow of Tbx6 mutants is functional and is perceived by the perinodal crown cells. Finally, in the Gdf1 rescue experiment, 25/26 of the rescued Tbx6+/−;node-Tg embryos showed left-sided Pitx2 expression. Because Tbx6 mutant embryos have randomized laterality, these results point to an additional role for Tbx6 further downstream in the genetic cascade that prevents the asymmetric expression of Nodal, Lefty2 and Pitx2 in the left LPM of mutant embryos, a role separate from its effects on nodal cilia and perinodal expression of Nodal.

**Tbx6 is upstream of Gdf1**

Gdf1 expression is critical for the long-range action of Nodal in activating asymmetric expression of Nodal in LPM (Tanaka et al., 2007). We showed that Tbx6 homozygous mutants do not express Gdf1 in the perinodal region but that a node-Tg transgene that expresses Gdf1 bilaterally in the perinodal region, could partially rescue Pitx2 expression in the left LPM/IFT of Tbx6 homozygous mutants. There are several possible reasons why rescue is not complete. First, in the transgene, Gdf1 is driven by a node-specific Nodal enhancer, which was shown to partially rescue left-sided Pitx2 expression in the LPM/IFT of Gdf1 mutants where 2/6 embryos showed relatively normal Nodal expression and 4/6 showed a restricted pattern of expression (Tanaka et al., 2007) indicating that the transgene did not restore full function. Secondly, the low level of Nodal expression in the perinodal region of Tbx6 mutants may compromise transduction of the signal, rendering rescue less likely.

The rescue experiment indicates that the absence of expression of left-specific genes in the LPM of Tbx6 homozygous mutants is due to the lack of Gdf1 perinodal expression. Although Tbx6 regulates luciferase reporter activity from the Gdf1 promoter, it does not do so through the putative Tbx6 binding sites we identified within 2 kb of the Gdf1 promoter region and thus Tbx6 may regulate Gdf1 expression through additional binding sites not detected in this analysis. The lack of a demonstrable genetic interaction in Tbx6+/−; Gdf1+/− and Tbx6+/−;Gdf1−/− compound heterozygous embryos indicates that even a reduced amount of Tbx6 protein is sufficient to drive perinodal Gdf1 expression.

In summary, the Tbx6 mutation impinges on several different components of the left-right axis determination pathway by affecting development of the node and nodal cilia, decreasing the level of Nodal expression in the perinodal region, and eliminating asymmetric Ca^{2+} signaling at the node (Hadjantonakis et al., 2008).
In this study, we have shown that in spite of these abnormalities, asymmetric gene expression indicative of a leftward nodal flow is still present and that Tbx6 additionally regulates expression of perinodal Gdf1, resulting in failure of transduction of the asymmetric signal to the LPM in mutant embryos and ultimately resulting in the randomization of laterality phenotype. It is not clear whether disruptions of node and nodal cilia and lower perinodal Nodal expression contribute to the laterality phenotype of Tbx6 mutants but it is interesting that Ca2+ signaling appears to be uncoupled from asymmetric gene expression at the node, indicating that disruptions of the nodal flow may differentially affect these two events.

**MATERIALS AND METHODS**

**Mice, embryo collection and ISH**

The null allele, Tbx6<sup>m276</sup> (referred to as Tbx6<sup>−</sup>) (Hadjantonakis et al., 2008) was maintained on a mixed 129 and ICR (Taconic) background. The null allele Gdf1<sup>m15/j</sup> (B6;129- Gdf1<sup>m15/j</sup>; The Jackson Laboratory, Bar Harbor, USA. Stock No. 004425; referred to as Gdf1<sup>−</sup>) is a targeted deletion of the protein coding region (Rankin et al., 2000). The node-Tg transgene contains Gdf1 cDNA linked to IRES-LacZ under the control of the node-specific enhancer (NDE) of Nodal which results in restricted expression of Gdf1 bilaterally in the perinodal region (Tanaka et al., 2007). Tbx6<sup>rib vertebrae</sup> (Tbx6<sup>−/−</sup>) (B6.L-Tbx6<sup>−/−</sup>; The Jackson Laboratory Stock No. 001052) is a hypomorphic allele (Watabe-Rudolph et al., 2002). Mice containing a null allele of Pak2 (referred to as Pak2<sup>−</sup>) were obtained by crossing the Pak2<sup>m1.17tw</sup> conditional allele [B6.129X1(Cg)-Pkd2<sup>m1.17tw</sup>/J] (Garcia-Gonzalez et al., 2010). All genotypes were maintained on mixed genetic backgrounds. To collect embryos for the epistasis experiment, a three-way cross was used to produce double-heterozygous Tbx6<sup>−/−</sup> Pak2<sup>−/−</sup> mice with or without the Gdf1<sup>−</sup> transgene, which were mated together to produce embryos with all combinations of genotypes.

Embryos were recovered between E7.5 and E8.5 (E0.5 denotes noon of the day of a vaginal plug) in phosphate buffered saline (PBS) containing 0.2% albumin bovine serum (Sigma-Aldrich), and staged by morphology (Downs and Davies, 1993); some were scored for placement of the tail, placenta, vitelline vessels and the direction of heart looping. Yolk sacs were used for PCR genotyping with primers for Tbx6<sup>−</sup>, Tbx6<sup>+</sup>, Gdf1<sup>−</sup>, node-Tg and Pak2 (Table S3). Alternatively, Tbx6<sup>−</sup> embryos were genotyped by fluorescence intensity which is proportional to the number of Tbx6<sup>−</sup>-alleles (Hadjantonakis et al., 2008). Embryos for ISH were fixed overnight in 4% pararmaldehyde in PBS at 4°C, dehydrated in 100% methanol and stored at −20°C until being processed for ISH using antisense RNA probes (Wilkinson, 1998). All animal protocols were approved by the Columbia University Medical Center Institutional Animal Care and Use Committee.

**EMSA**

A coupled in vitro transcription/translation system with rabbit reticulocyte lysate (TNT® Promega, Madison, USA) was used to make full-length Tbx6 protein for DNA binding studies (White and Chapman, 2005). 1 µg of Tbx6<sup>+</sup> cDNA cloned into a pET21a expression vector (Novagen, Sigma-Aldrich) was incubated in 50 µl of rabbit reticulocyte lysate for 1 h at 30°C. Fluorescent probes were generated by attaching a linker sequence 5′-GTAGAGACTGTG-3′ to the 3′ end of the reverse complement of five putative Tbx6 binding sites and mutant versions of each, created by mutating the core sequence of the T-box binding element. To create a double-stranded, fluorescently labeled DNA probe for EMSA, a fluorescently labeled oligonucleotide, Tbx6 linker: 5′-Cy5-GTAGAGACTGTG-3′, was annealed to the linker sequence on each of the reverse complement oligonucleotides using Klenow polymerase to fill in the remaining sequence. Three microliters of lysate containing either the Tbx6 expression construct or empty vector was incubated with 0.3 ng of each fluorescently labeled probe in binding buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 10 µM ZnCl<sub>2</sub>, 6% glycerol, 200 µg of bovine albumin/ml, and 50 µg of poly(dC-poly(dC)-ml) (Gebelien and Urrutia, 2001) for 20 min at room temperature. Each binding reaction was then loaded onto a 4% polyacrylamide gel and run at 120 V at 4°C. Fluorescent probe alone was loaded as a negative control. The gel was then vacuum dried and imaged using a Typhoon TRIO variable mode imager (Amersham Biosciences, Little Chalfont, UK).

**Cell culture and luciferase assays**

NIH-3T3 cells (ATCC) were cultured in DMEM (Gibco) with 10% FBS (HyClone, Thermo Fisher Scientific), 1% Penicillin/Streptomycin (Gibco), 1% non-essential amino acids (Gibco), 1% sodium pyruvate (Gibco). Cells were split at 80% confluency and plated at a concentration of 200,000 cells per ml for transfection with a luciferase reporter containing the promoter region of Gdf1, a luciferase reporter containing mutated putative Tbx6 binding sites, and Tbx6 cDNA cloned in-frame into the expression vector pCMV-Tag2a that inserts a FLAG tag at the 5′ end of Tbx6 protein. Transfection was achieved by PEI-based transfection (Elhardt et al., 2006). Cells were collected after 48 h and lysed in cold NP40 Vanadate lysate buffer containing protease inhibitor cocktail (Sigma-Aldrich) and 1 mM phenylmethylsulfonyl fluoride for 20 min. Cells were centrifuged and protein in the supernatant was quantified by Bradford reagent (Bio-Rad) and used for western blotting. Then, 10 µg of protein/sample/lanne was run on a 4% polyacrylamide gel and transferred onto polyvinylidene difluoride membrane. Western Blots were blocked in 4% milk for 30 min at room temperature and incubated with mouse anti-FLAG antibody at 1:200 (Sigma-Aldrich) overnight at 4°C. Blots were washed with PBS containing 0.1% Tween-20 and anti-mouse horseshadish peroxidase (The Jackson Laboratory) was added at a concentration of 1:10,000 for 1 h. Blots were visualized with ECL Substrate (Thermo Fisher Scientific).

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

Conceptualization: D.C., V.E.P.; Methodology: D.C., V.E.P.; Formal analysis: D.C., V.E.P.; Investigation: D.C., V.E.P.; Resources: H.H., V.E.P.; Writing - original draft: D.C., V.E.P.; Writing - review & editing: D.C., H.H., V.E.P.; Visualization: H.H., V.E.P.; Supervision: V.E.P.; Project administration: V.E.P.; Funding acquisition: V.E.P.

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**Supplementary information**

Supplementary information available online at http://bio.biologists.org/lookup/doi/10.1242/bio.032565.supplemental

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