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Tiki1 is required for head formation via Wnt cleavage-oxidation and inactivation

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SUMMARY

Secreted Wnt morphogens are signaling molecules essential for embryogenesis, pathogenesis, and regeneration, and require distinct modifications for secretion, gradient formation and/or activity. Whether Wnt proteins can be post-translationally inactivated during development/homeostasis is unknown. Here we identify, through functional cDNA screening, a transmembrane protein Tiki1 that is expressed specifically in the dorsal Spemann-Mangold Organizer and is required for anterior development during *Xenopus* embryogenesis. Tiki1 antagonizes Wnt function in embryos and human cells via a TIKI homology domain that is conserved from bacteria to mammals, and acts likely as a protease to cleave eight amino terminal residues of a Wnt protein, resulting in oxidized Wnt oligomers that exhibit normal secretion but minimized receptor-binding capability. Our findings identify a Wnt-specific protease that controls head formation, reveal a mechanism for morphogen inactivation through proteolysis-induced oxidation-oligomerization, and suggest a link between Wnt palmitoylation and evasion of oxidizing inactivation. TIKI proteins may represent potential therapeutic targets.

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ACCESSION NUMBERS Sequences of human *TIKI1, TIKI2, Xenopus Tiki1, Tiki2,* zebrafish *Tiki1, Tiki2, C. elegans Tiki, Nematostella Tiki,* and *Amphimedon Tiki* have been deposited in GenBank under accession numbers JQ653415, JQ653416, JQ653417, JQ653418, JQ653419, JQ653420, JQ653421, JQ653422, and JQ653423, respectively.

SUPPLEMENTAL INFORMATION Supplemental Information includes Extended Experimental Procedures, seven figures and four tables and can be found with this article on line at

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INTRODUCTION

The Wnt family of secreted proteins control embryogenesis, homeostasis, and regeneration, and Wnt signaling deregulation causes birth defects, cancer, osteoporosis and other diseases (Clevers, 2006; Logan and Nusse, 2004; MacDonald et al., 2009; Moon et al., 2004). As prototypic morphogens Wnt proteins exhibit short and long range signaling properties in a concentration gradient field (Hausmann et al., 2007; Strigini and Cohen, 2000; Zecca et al., 1996). Mechanisms that regulate Wnt biogenesis and gradient have not been fully defined. Critical for Wnt biogenesis/function are post-translational modifications including N-glycosylation and lipidation, as best exemplified for mouse Wnt3a. O-acylation of serine 209 (S209) and N-glycosylation appear to be required for Wnt3a secretion (Komekado et al., 2007; Takada et al., 2006), whereas palmitoylation of cysteine 77 (C77) is important for Wnt3a activity (Willert et al., 2003). These modifications have profound influence on Wnt exocytic trafficking, extracellular distribution and/or receptor binding (Cong et al., 2004; Franch-Marro et al., 2008; Komekado et al., 2007; Kurayoshi et al., 2007; Takada et al., 2006; Willert et al., 2003; Zhai et al., 2004). Whether Wnt proteins are post-translationally modified and inactivated is unknown.

Canonical Wnt signaling plays a central role in anterior-posterior (AP) patterning (De Robertis and Kuroda, 2004; Niehrs, 2004; Petersen and Reddien, 2009). Wnt engagement of receptors including the Frizzled (Fz) serpentine receptor and the single-span LDL receptor-related protein 6 (LRP6) induces the stabilization of transcription co-activator β-catenin (He et al., 2004; MacDonald et al., 2009), thereby activating gene expression for AP patterning. In *Xenopus* embryos an AP gradient of Wnt signaling occurs with the highest level posteriorly, and the dorsal Spemann-Mangold Organizer promotes head development via secreting Wnt antagonists such as secreted Fz-related proteins (sFRPs) and Dickkopf-1 (Dkk1), which bind to and inhibit Wnt/Fz or LRP6 to keep Wnt signaling low anteriorly (De Robertis and Kuroda, 2004; Harland and Gerhart, 1997; Niehrs, 2004). Here we describe an Organizer-specific transmembrane protein, Tiki1, which is required for head formation via cleavage and inactivation of Wnt ligands.

RESULTS

Expression cloning of Tiki1

We performed a functional cDNA expression screen in *Xenopus* embryos to identify genes involved in AP patterning/Wnt signaling. One isolated cDNA caused head enlargement and was named *Tiki1* (Tiki refers to a large headed humanoid in Polynesian mythology) (Figure 1A). *Tiki1* encodes a type I transmembrane protein of 508 residues with an amino-terminal signal peptide, an ectodomain, and a transmembrane domain at the carboxyl terminus (Figure 1B). Tiki1 is the founding member of a new conserved protein family, and a Tiki1 homologue is encoded in the genome of sponge *Amphimedon queenslandica* and sea anemone *Nematostella vectensis* and is likely in the common ancestor before invertebrates and vertebrates diverged (Figures 1C and S1). Most vertebrates including *Xenopus*, zebrafish and human have two genes, *Tiki1* and *Tiki2* (Figures 1C and S1). Tiki proteins do not harbor known motifs, but exhibit a conserved ectodomain of 340 residues, referred to as the TIKI domain, which shares 25% identity/40% similarity with putative bacterial gumN proteins of unknown functions (Figures 1C and S2A).

Tiki1 antagonizes Wnt function

Head enlargement by Tiki1 overexpression phenocopies that by Dkk1 (Figure 1A), a Wnt/LRP6 antagonist (He et al., 2004)**.** Indeed Tiki1 antagonized Wnt function and inhibited axis duplication and induction of Xnr3 (a Wnt target gene) by *Xenopus* wnt8 (Xwnt8), but not by

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β-catenin or Dishevelled, another Wnt downstream component, and importantly not by a constitutively active LRP6ΔN (Tamai et al., 2004) (Figures 1D and 1E). Thus Tiki1 likely acts at or upstream of Wnt receptors. Tiki1 did not affect signaling by secreted factors such as Nodal, a TGF-β (transforming growth factor-β) protein, or bFGF (basic fibroblast growth factor) (Figures 1F and 1G), attesting its specificity. *Xenopus* Tiki2, and human TIKI1 and TIKI2 also behaved as Wnt antagonists in embryos (not shown), HEK293T cells (Figure S2B) and mouse L cells (below). TIKI1N or TIKI2N, which is a secreted form of the respective ectodomain (Figures 1B and S1), was fully functional and interchangeable with TIKI1/2 in embryos (not shown) and cultured cells (Figure S2C).

Organizer expression of Tiki1

RT-PCR revealed that *Tiki1* mRNA becomes detectable at stage 9 and is prominently expressed from stage 10 (early gastrula) to stage 30 (tadpole) (Figure 2A), and interestingly is restricted to the dorsal segment of the gastrula (Figure 2B). At stage 10 *Tiki1* is expressed exclusively in the dorsal blastopore lip, the Organizer, in a pattern similar to that of *Dkk1* (Figures 2C and 2D). Longitudinal hemi-sectioning of the stage 10.5 embryo revealed that *Tiki1* is excluded from the dorsal margin of the Organizer and overlapped with *Dkk1* expression in the prechordal mesoderm and endomesoderm (Figures 2E and 2F). At stage 11 *Tiki1* and *Dkk1* are expressed in anterior prechordal plate (Figures 2G and 2H). By blastopore closure *Tiki1* expression becomes distinct from that of *Dkk1,* as *Tiki1* is restricted to the anterior midline while *Dkk1* displays a wing-shaped expression straddling *Tiki1* (Figures 2I and 2J). At early neurula *Tiki1* is expressed in the midline anterior to the tip of the notochord in cells of the endoderm and overlying neural ectoderm while *Dkk1* has a more broad expression pattern in the prospective ventral forebrain (Figures 2K and 2L). Thus *Tiki1* is zygotically expressed in the Organizer, in particular in the head organizer region responsible for anterior patterning.

Organizer formation requires maternal Wnt signaling (De Robertis and Kuroda, 2004; Harland and Gerhart, 1997). Ectopic Xwnt8 induced *Tiki1* and *Dkk1* expression ventrally, whereas a dominant negative TCF (T cell factor, a DNA-binding factor that mediates β-catenin signaling), ΔNTCF (Molenaar et al., 1996), suppressed the endogenous *Tiki1* and *Dkk1* dorsal expression (Figure 2M). LiCl, which stabilizes β-catenin thus dorsalizes embryos, induced *Tiki1* and *Dkk1* expression, while ultraviolet irradiation, which causes defective maternal Wnt signaling and ventralizes embryos, suppressed *Tiki1* and *Dkk1* expression (Figure 2M). Thus maternal Wnt signaling is necessary and sufficient for *Tiki1* and *Dkk1* expression in the Organizer.

Tiki1 is required for head development

We performed Tiki1 protein depletion using a Tiki1 morpholino antisense oligonucleotide (Tiki1MO), which specifically blocked the synthesis of the exogenously expressed Tiki1 (Tiki1-FLAG) in embryos (Figure 3A). Tiki1MO was unable to block the synthesis of HA*Tiki1, whose mRNA was engineered to lack the MO targeting sequence, attesting Tiki1MO specificity (Figure 3A). We injected 20ng of Tiki1MO into two dorsal blastomeres of 8-cell stage embryos. Strikingly roughly 50% of Tiki1MO-injected embryos developed anterior defects exhibiting loss of forebrain structures, including diminished or loss of the cement gland and eyes, or exhibiting cyclopic eyes fused at the midline (Figures 3B and 3C). HA*Tiki1 mRNA rescued Tiki1MO phenotypes (Figures 3B and 3C). Supporting a role of Tiki1 in Wnt inhibition during head formation, Dkk1 mRNA also rescued Tiki1MO anterior deficiency phenotypes (Figure S3A).

Tiki1MO suppressed the expression of head organizer genes including *Goosecoid (Gsc)*, *Lim1*, *Otx2*, and *Dkk1* in gastrula embryos (stages 10.5-11) (Figure 3D and Table S1).

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Tiki1MO also inhibited the expression of Organizer gene *Chordin*, but not of other Organizer or dorsal genes *Xnr3* and *Xnot* (Figure 3D and Table S1), which are not involved in head organizer function. Tiki1MO reduced *Gsc* expression in prechordal plate, the descendent of the head organizer, in neurula (Figure S3B and Table S1). HA*Tiki1 mRNA rescued Tiki1MO effects on all head organizer genes examined (Figure 3D and Table S1). Control MO did not affect any marker examined (Figure 3D and Table S1). Tiki1 MO did not affect *Gsc* expression prior to stage 10.5 (Figure S3C and Table S2), which relies on maternal Wnt signaling (Cho et al., 1991; Christian and Moon, 1993). Thus Tiki1 is required for maintenance of the head organizer via antagonizing zygotic Wnt signaling. Consistent with this, Tiki1 inhibited Xwnt8 posterizing effect when both were expressed dorsally and zygotically via injected plasmids (Figure S3D).

Tiki1 expression/function in the head organizer implies its role in anterior neural patterning. Tiki1MO injected into one dorsal-animal blastomere at the 8-cell stage suppressed at stage 16 the expression of forebrain markers *BF1* and *Otx2* and the midbrain marker *En2*, but not the posterior neural marker *Hoxb9*, and HA*Tiki1 mRNA rescued the expression of *BF1*, *Otx2*, and *En2* in Tiki1MO-injected embryos (Figure S3E and Table S3). Therefore Tiki1 is required for anterior neural patterning.

TIKI expression and function in human cells

Tiki genes exhibit specific expression during embryogenesis (Figure 2 and data not shown). We found, unexpectedly, that human HEK293T and HeLa cells, which are commonly used for Wnt signaling studies, express mRNAs for TIKI2, and TIKI1 plus TIKI2, respectively (Figure 4A and data not shown). Depletion of TIKI2 in HEK293T cells via siRNAs enhanced Wnt3a-induced reporter expression by up to 7 folds (Figure 4B), and the enhancement levels correlated with the extent of reduction of TIKI2 mRNA and the tagged TIKI2 protein by the siRNAs (Figures 4C and S4A); furthermore, the TIKI2 siRNA effect was rescued by ectopic TIKI1 expression (Figure 4D). Thus the endogenous TIKI protein(s) acts to restrain Wnt signaling in cultured human cells.

Wnt inactivation by Tiki proteins

We investigated how Tiki antagonizes Wnt signaling. Upon expression in HEK293T or HeLa cells, Tiki1/TIKI1 and TIKI2 were detected at the plasma and internal membranes via imaging and surface biotinylation (Figures 4E-4G). We stably expressed TIKI2 in L cells or Wnt3a-expressing L cells (which expressed TIKI1 poorly ectopically). TIKI2 inhibited β-catenin stabilization in Wnt3a-producing cells (Figure 4H, lanes 5 and 6), but apparently not in cells treated with Wnt3a conditioned media (CM) (Figure 4H, lanes 3 and 4). Interestingly Wnt3a produced in L cells was secreted normally into CM in the presence of TIKI2, but exhibited faster electrophoretic migration prior to (in whole cell lysates) and after secretion (in CM) (Figure 4I). Similar results were observed for Wnt3a in HEK293T cells that co-express TIKI1 or TIKI2 (Figure S4B). Wnt3a secreted from TIKI-expressing cells exhibited minimal activity, as it neither induced the Wnt-responsive reporter (Figure 4J), phosphorylation of LRP6 and Dishevelled, nor stabilized β-catenin (Figure 4K). Revealingly Wnt3a secreted from TIKI2-expressing cells bound to neither Fz nor LRP6 (Figure 4L). Thus TIKI proteins result in a Wnt3a modification that minimizes Wnt3a activity but not secretion.

Elimination of Wnt hydrophobicity by Tiki proteins

Palmitoylation at C77 appears to be required for Wnt3a binding to Fz and LRP6, and the inactivity of Tiki-modified Wnt3a is reminiscent of Wnt3a(C77A), which harbors alanine substitution of C77 and lacks palmitoylation (Cong et al., 2004; Komekado et al., 2007; Willert et al., 2003). In a detergent-aqueous phase separation assay Wnt3a partitions in

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Triton X-114 detergent whereas Wnt3a(C77A) is soluble aqueously (Komekado et al., 2007; Willert et al., 2003). Wnt3a from TIKI1/2-expressing cells partitioned exclusively in the aqueous phase (Figures 5A and S4C), further resembling Wnt3a(C77A). Unexpectedly metabolic labeling detected little difference in Wnt3a palmitate incorporation with or without TIKI2 (Figures 5B and S4D). Wnt3a(C77A), and in fact Wnt3a(C77A/S209A) that harbors alanine substitutions at both C77 and S209 and lacks lipidation at these two residues, were modified by TIKI2 as judged by electrophoretic mobility changes (Figures S4E and S4F), and the residual autocrine signaling activity of Wnt3a(C77A) was inhibited by TIKI2 (Figure S4G). TIKI2 also modified Wnt3a(N87Q/N298Q), which has glutamine substitutions at the two glycosylated asparagines and lacks N-glycosylation (Komekado et al., 2007) (Figure S4F). Thus despite elimination of Wnt3a hydrophobicity, Tiki modification of Wnt3a appears to be unrelated to C77 or S209 acylation or N-glycosylation.

Wnt amino-terminal cleavage by Tiki proteins

Wnt3a mutants deleted of the amino-terminal region were invariably resistant to Tiki modification (Figure S4H). Edman sequencing of TIKI2-modified Wnt3a revealed that eight amino-terminal residues, SYPIWWSL, had been removed from the mature Wnt3a (i.e., after signal peptide removal) (Figure 5C). A quantitative mass spectrometry (MS) approach was taken to further analyze this amino-terminal processing. Wnt3a was expressed alone or with TIKI2 in light or heavy SILAC (*s*table *i*sotope *l*abeling by *a*mino acids in *c*ell culture) medium (Ong et al., 2002), and analyzed for MS using FLEXIQuant quantification (Singh et al., 2009) (Figure S5A). The relative peptide peak ratios of Wnt3a with and without TIKI2 confirmed cleavage of the 8 residues in TIKI2-modified Wnt3a, and also uncovered two additional cleavage sites of lesser abundance, which are 7 and 8 residues downstream of the main cleavage site (Figures 5C, S5B and S5C). The relative peptide peak ratios for the remaining Wnt3a with or without TIKI2 were largely unperturbed, suggesting that TIKI2 might not affect the rest of Wnt3a protein (Figure S5B and Table S4). We generated HA-Wnt3a, which harbors the HA tag after the signal peptide. Remarkably TIKI1 and TIKI2 resulted in removal of the HA epitope while leaving the rest of Wnt3a apparently intact (Figure 5D). The HA tag had no influence on Wnt3a cleavage by TIKI2 (Figure S6A). TIKI2 did not cleave the HA tag in HA-R-spondin1 (Rspo1, (Wei et al., 2007), a secreted Wnt agonist that harbors the HA tag after the signal peptide (Figure 5E). siRNA knockdown of the endogenous TIKI2 reduced the cleavage of HA-Wnt3a in HEK293T cells, as shown by increased retention of the HA epitope (Figure 5F). Thus Tiki resulted in amino terminal cleavage of Wnt3a (but not Rspo1), consistent with data from Edman sequencing and SILAC-FLEXIQuant analysis. To confirm TIKI cleavage specificity, we altered the two residues straddling the main and minor cleavage sites in Wnt3a (Figure 5C). These HA-Wnt3a mutants, Wnt3a(S25D/L26D) and Wnt3a(S25D/L26D/S33D/S34D), were partially and completely resistant to TIKI2 proteolysis, respectively (Figure 5G, lanes 2, 4, and 6), demonstrating TIKI2 acting through these residues. We then generated Wnt3aΔN, which lacks the exact 8 residues removed by TIKI2 (Figure 5C). Wnt3aΔN was secreted normally but partitioned in the aqueous phase and exhibited minimal activity (Figures 5H and 5I), thereby behaving like Tiki-modified Wnt3a (Figure 4J). Therefore Tiki promotes cleavage of Wnt3a amino terminus, which has an essential role in Wnt3a activity. Supporting this notion, Wnt3a(S25D/L26D/S33D/S34D) was inactive despite secreting normally (Figures S6B and S6C, the bottom panel).

Tiki cleavage of multiple Wnt proteins

Tiki1/2 inhibits Xwnt8 in embryos (Figures 1, S3D and data not shown). TIKI2 cleaved Xwnt8, as shown by removal of the amino terminal HA tag (after the signal peptide) from HA-Xwnt8-FLAG, which also had a carboxyl terminal FLAG tag (Figure 5J). MS analyses of Xwnt8-Myc or HA-Xwnt8-FLAG cleaved by TIKI2 suggested identically two amino

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terminal cleavage sites, resulting in removal of 17 and 20 residues of mature Xwnt8, respectively (Figure 5C and data not shown). TIKI2 also cleaved HA-Wnt5a at the amino terminus (Figure 5K). The low amount of Wnt5a production has hindered identification of the cleavage site(s). TIKI2 did not cleave HA-Xwnt11-FLAG (Figure 5L), implying Wnt specificity.

Amino terminal residues are highly variable among Wnt proteins despite conservation among paralogs in vertebrates. Consequently TIKI2 cleavage sites in Wnt3a and Xwnt8 do not reveal recognizable consensus motifs (Figure 5C). To glimpse into this apparent paradox, we found that TIKI2, but not KRM2 (Kremen2, (Mao et al., 2002), bound to Wnt3a, Xwnt8, and Wnt5a, but not Rspo1 (Figure S6D-F and data not shown). This was not unanticipated since enzyme-substrate pairs often show interaction. Intriguingly TIKI2, but not KRM2, also bound to Xwnt11 (Figure S6G). We speculate that TIKI may interact with conserved motifs within Wnt proteins, thereby orientating itself for cleaving specific Wnt amino terminus.

Tiki is likely a metalloprotease

TIKI2N was sufficient for Wnt3a inhibition and cleavage in cells (Figures 5, S2C, and S4). We purified via a tandem affinity procedure recombinant TIKI2N, which upon *in vitro* incubation with purified HA-Wnt3a, triggered Wnt3a cleavage and the HA disappearance (Figures 5N and 5O). KRM2N, which corresponds to Kremen2 ectodomain, or TIKI2NΔ, a deletion mutant that lacks a TIKI-domain segment and neither modified Wnt3a nor inhibited Wnt signaling in cells (Figures 5M and S6H), did not cleave HA-Wnt3a *in vitro* (Figure 5O). MS analyses of recombinant WNT3A cleaved by TIKI2N *in vitro* indicated cleavage at identical (major and minor) amino terminal sites as observed in cells (data not shown). These results suggest that Tiki is, or is associated with, a Wnt protease.

We characterized TIKI protease activity *in vitro*. TIKI2N cleavage of HA-Wnt3a became obvious at 2 hours, and exhibited preference for neutral to slightly basic pH (pH 7-9) (Figures S7A and S7B). We applied protease inhibitors to survey which protease family TIKI belongs to. TIKI2N was neither sensitive to Bestatin, an aminopeptidase inhibitor, nor to a mixture of inhibitors for serine, cysteine, aspartic proteases and aminopeptidases (Figures 5P, S7C and S7D). Remarkably, TIKI2N was inhibited by a metalloprotease inhibitor, 1,10-phenanthroline, which is a divalent metal chelator and was applied at concentrations 100 folds lower than recommended doses (Figure 5P). TIKI2N was also inhibited, albeit mildly, by EDTA, another metalloprotease inhibitor and divalent metal chelator (Figure S7E) (Barrett, 1998; Overall and Blobel, 2007). Metalloproteases are defined by their dependence on divalent metals as co-factors. TIKI2N cleavage of HA-Wnt3a *in vitro* was enhanced by Co^{2+} or Mn^{2+} (Figure 5Q, lanes 4 and 12), but not by Ni^{2+}, Cu^{2+}, or Zn^{2+}, which in fact inhibited TIKI2N (Figure 5Q, lanes 6, 8, and 10). Importantly, Co^{2+} or Mn^{2+} restored TIKI2N enzymatic activity that was inhibited by 1,10-phenanthroline (Figure 5R, lanes 6 and 8). Thus Tiki is likely a Co^{2+}- or Mn^{2+}-dependent metalloprotease.

Wnt oxidation-oligomerization induced by Tiki cleavage

Tiki-induced elimination of Wnt3a hydrophobicity (Figures 5A and S4C) was puzzling but intriguing. We unexpectedly found that Tiki-modified Wnt3a, but not control Wnt3a, existed in CM as large (>400 kD) soluble oligomers, which were linked by inter-molecular disulfide bonds and massive in size since a significant fraction showed minimal migration in gel (Figure 6A). This oxidized Wnt oligomerization was secondary to Tiki cleavage, since Wnt3aΔN formed, without Tiki expression, similar large soluble oligomers that could be reduced (Figure 6B), as did Wnt3a(S25D/L26D/S33D/S34D), which is also inactive (Figure S6C). Interestingly oxidized Wnt3a oligomers partitioned exclusively aqueously whereas

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monomeric (or non-covalently oligomeric) Wnt3a partitioned in detergent (Figure 6C), implying that lipid adducts may have been buried inside Wnt oligomers. Control Wnt3a exhibited traces of oxidized oligomers (Figures 6A-C), which contained intact Wnt3a as demonstrated by the amino terminal HA tag (Figure S7F). Thus Wnt3a may have intrinsic oxidation-oligomerization propensity, which Wnt3a amino terminus acts to minimize. However, we cannot rule out that the trace amount of oxidized oligomers of control Wnt3a may be due to overexpression.

Tiki-modified Wnt3a, Wnt3aΔN, and Wnt3a(C77A) bare strong similarity in hydrophilic behavior and inactivity. Remarkably, Wnt3a(C77A) also formed oxidized oligomers in CM regardless of Tiki expression (Figure 6D). Although it is unknown whether these various oxidized Wnt3a oligomers were formed via disulfide bonds among the same cysteines, Wnt3a C77 palmitoylation may also be required for minimizing Wnt3a oxidation-oligomerization, a role shared by Wnt3a amino terminus that is removable by Tiki. However, while Tiki-modified Wnt3a and Wnt3aΔN are severely impaired in autocrine and paracrine signaling (Figures 4J, 5H, S2B and S2C), Wnt3a(C77A) retains residual autocrine signaling activity (Figure S4G) (Willert et al., 2003), implying quantitative or qualitative difference in roles that Wnt3a amino terminus and palmitoylation play in maintaining Wnt3a activity.

We examined where Wnt oligomers were formed during biogenesis. Surprisingly in whole cell lysates there was little difference among Wnt3a, Wnt3a(C77A), and Wnt3a(S209A), regardless of TIKI2 cleavage: each existed as monomers (or non-covalent oligomers) and heterogeneous oxidized oligomers from likely dimers to larger multimers (Figure 6E). These results may be consistent with our suspicion that during biogenesis Wnt3a has intrinsic oxidizing propensity, which is minimized by Wnt3a amino terminus and C77 palmitoylation during/after secretion, leaving only traces of oxidized oligomers in CM. But for TIKI-cleaved Wnt3a or Wnt3a(C77A), this propensity may be exacerbated during/after secretion, resulting in massive oxidized oligomers in CM. Wnt3a(S209A) was not secreted as reported (Figure S7G; (Takada et al., 2006).

Tiki can act in both Wnt-producing and Wnt-responding cells

Cellular localization (Figures 4E-4G) suggests that Tiki may act on secretory and plasma membranes. Consistent with both scenarios, Tiki1N (Figure S2C) and Tiki1N-KDEL, which harbors the ER-retention KDEL sequence at Tiki1N carboxyl terminus and was hardly secreted (Figure S7H), each potently inhibited Wnt signaling (Figure S6H). Tiki showed inhibition in Wnt-producing cells, but apparently not in Wnt-responding cells (Figure 4H). We were concerned however that excessive Wnt3a added acutely might have activated signaling prior to, or overwhelmed, Tiki function. We thus investigated this issue under more physiological settings in embryos. We performed Wnt-responsive reporter assays via Tiki overexpression or knockdown. Firstly we injected two animal blastomeres separately with Xwnt8 mRNA or S01234-luciferase DNA (Figure 7A), which is driven by the promoter of the *Siamois* gene, a direct Wnt target (Brannon et al., 1997). Animal blastomeres were chosen as their progenies do not express Tiki1 (Figure 2). Xwnt8 induced expression of the reporter (Figure 7A), but not of S0-luciferase in which TCF-binding sites were mutated (Brannon et al., 1997) (data not shown). Co-injection of Xwnt8 with Tiki1, but not LDLR, inhibited the reporter (Figure 7A). Thus Tiki1 could inhibit Wnt activity in Wnt-producing cells as seen in mammalian cells. We then altered the co-injection scheme by injecting Xwnt8 in one blastomere and injecting Tiki1 (or LDLR) mRNA plus S01234-luciferase in a neighboring blastomere (Figure 7B). Tiki1, but not LDLR, inhibited Xwnt8-induced reporter in Wnt-responding cells (Figure 7B).

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We addressed the same question complementarily via Tiki1MO knockdown in dorsal blastomeres whose progenies express Tiki1 endogenously. We co-injected an Xwnt8 plasmid (transcribing Xwnt8 after mid-blastula transition) plus Tiki1MO or control MO into a blastomere (i.e., to knock down Tiki1 in Xwnt8-producing cells), and injected S01234-luciferase into the neighboring blastomere (Figure 7C). Alternatively we injected the Xwnt8 plasmid into a single blastomere, and co-injected S01234-luciferase plus Tiki1MO or control MO into the neighboring blastomere (i.e., to knock down Tiki1 in Xwnt8-responding cells, Figure 7D). Xwnt8 induced S01234-luciferase, but not S0-luciferase, and the XWnt8 response was enhanced by Tiki1MO under either condition (Figures 7C and 7D), Thus the endogenous Organizer Tiki1 acts in both Wnt-producing and Wnt-responding cells.

We further monitored how Tiki1 inhibits Wnt-induced nuclear β-catenin accumulation. We injected Xwnt8 plus RFP (red fluorescent protein) mRNA into an animal blastomere, and injected Tiki1 or LDLR mRNA plus fluorescein dextran (FLD) into a neighboring blastomere (Figure 7E). Thus Xwnt8-expressing cells were RFP^+ (red), whereas Wnt-responding cells that expressed Tiki1 or LDLR were FLD^+ (green), and naive cells from uninjected blastomeres lacked either fluorescence (Figures 7G, 7H, 7K and 7L). Nuclear β-catenin (blue) was observed at stage 9 in Xwnt8-expressing cells (red) due to autocrine signaling (arrows, Figure 7I), and in cells that expressed LDLR (green) in response to XWnt8 (arrowheads, Figure 7I). By contrast, much fewer Tiki1-expressing cells (green, Figure 7K) exhibited nuclear β-catenin (arrowheads, Figure 7M). Quantification showed 75% of LDLR-expressing and naive cells versus 30% of Tiki1-expressing cells displaying nuclear β-catenin (Figure 7F), supporting that Tiki1 functions in Wnt-responding cells. We propose that Tiki functions in Wnt-producing cells via acting in the secretory pathway and at the plasma membrane, and in Wnt-responding cells via acting at the plasma membrane.

DISCUSSION

We identified a new family of Tiki proteins that inactivate Wnt morphogens through proteolytic cleavage. Tiki1 is expressed in the Spemann-Mangold Organizer and is required for head formation in *Xenopus* embryos. In the absence of Tiki1, expression of all head organizer genes examined is diminished, resulting in defective head organizer function, which in turn causes deficiency in anterior neural patterning. Tiki1 appears to be required for Organizer maintenance, which is antagonized by zygotic Wnt signaling, but not for initiation of Organizer formation, which requires maternal Wnt signaling. Tiki1 as a founding “Wnt inactivator” thus joins known Organizer-specific Wnt antagonists such as Dkk1 and Frzb/sFRP3, and acts to ensure a “Wnt-free” zone for anteriorization. Tiki1 functions in both Wnt-producing and Wnt-responding cells, thereby inactivating Wnt proteins made by the organizer or by neighboring or posterior regions**.** Organizer Tiki1 expression is induced by maternal Wnt signaling, reflecting a negative feedback loop that is common in morphogen signaling. Tiki-mediated Wnt inactivation may exist before the split of invertebrate and vertebrate lineages since a Tiki homolog is encoded in the genome of sponge *Amphimedon* and of sea anemone *Nematostella*. Tiki proteins share homology with putative bacterial gumN proteins of unknown functions, and thus represent the first example of Wnt components with a prokaryotic origin and further imply potential mechanisms for gumN and related TraB proteins.

Tiki is likely, or is associated with, a divalent metal (Co^{2+} or Mn^{2+})-dependent metalloprotease that cleaves the amino terminus of Wnt proteins, despite baring no recognizable homology to known proteases. TIKI2 cleaves Wnt3a, Xwnt8, and Wnt5a, but not Xwnt11, implying broad yet stringent Wnt specificity. The Wnt amino terminus is highly variable among one another but well conserved among vertebrate paralogs, as such TIKI2 cleavage sites in Wnt3a and Xwnt8 do not reveal a recognizable consensus. As TIKI2

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interacts with each Wnt tested including Xwnt11, TIKI may bind to a conserved motif within Wnt proteins and then cleave Wnt amino termini selectively. Tiki neither inhibits signaling by Nodal/TGF-β or bFGF, nor cleaves Rspo1. Importantly TIKI fails to cleave a Wnt3a mutant with missense alterations at the proteolytic sites, further reflecting selectivity for peptide bond hydrolysis.

Tiki cleavage results in Wnt3a oxidation and oligomerization through inter-Wnt disulfide bonding. These soluble Wnt3a oligomers exhibit drastically altered biophysical and biochemical properties including the loss of hydrophobicity and of receptor-binding, which are fully recapitulated by Wnt3aΔN. Thus the Wnt amino terminus has a critical role in preventing Wnt oxidation-oligomerization, a feature exploited by Tiki1 during anterior patterning. This feature may also relate to Wnt lipid modifications, which are exemplified by Wnt3a C77 palmitoylation and S209 acylation and have distinct roles in Wnt biogenesis/ function. S209 acylation is essential for Wnt3a secretion (Takada et al., 2006) due to its involvement in Wnt3a-binding to the Wntless transporter (Banziger et al., 2006; Coombs et al., 2010). Wnt3a C77 palmitoylation is required for activity but not secretion (Willert et al., 2003) via an yet unclear mechanism. Our study suggests a potential link between palmitoylation and Wnt folding. Wnt3a(C77A), which lacks palmitoylation and is commonly used in Wnt studies, shares similar behaviors with Tiki-modified Wnt3a/ Wnt3aΔN, as they are all hydrophilic, inactive, and form in CM oxidized oligomers, which in fact may explain why each fails to bind to Fz or LRP6. We do not discount that C77 palmitoylation and Wnt3a amino terminus may be involved in binding to both Fz and LRP6 simultaneously, but such a scenario appears to be more convoluted. Therefore C77 palmitoylation, like Wnt3a amino terminus, is likely required for keeping Wnt3a from oxidation-oligomerization during biogenesis. A new study shows that a mutant *Drosophila* Wingless (Wg), Wg^{C93A}, which has an alanine substitution at C93 (equivalent to Wnt3a C77), is secreted but forms large-sized (>1.3M Dalton) soluble aggregates (Mulligan et al., 2012). Although it is to be determined whether Wg^{C93A} aggregates are formed by inter-Wg disulfide bonds like Wnt3a(C77A) oligomers, a conserved role of palmitoylation in preventing oligomerization may be shared between Wg and Wnt3a. We note that our findings do not exclude the possibility if active Wnt proteins are non-covalent oligomers or monomers.

Oxidized oligomers formed by Tiki-modified Wnt3a or Wnt3a(C77A), which harbors dual lipids or a single lipid, are soluble aqueously (Willert et al., 2003; this study), and in fact exhibit strict correlation with hydrophilic behavior, in sharp contrast to Wnt3a monomers. Thus these inactive Wnt oligomers are unlikely misfolded/denatured aggregates, but rather may represent an alternative conformation that buries the lipid(s) inside, thereby behaving hydrophilic. It is intriguing how Tiki removal of eight amino terminal residues, which do not contain a cysteine, exacerbates inter-Wnt3a oxidation, which seems to be prevalent during biogenesis (Figure 6E). Among important questions are the specificity and mechanism of Tiki1/2 towards Wnt proteins, whether Tiki cleavage regulates other Wnt pathways, how Wnt amino terminus and palmitoylation maintain Wnt in the active state, and whether oxidized Wnt oligomers, given the reversible nature of oxidation, can be reduced/ functionally reactivated by reducing enzymes or disulfide isomerases. Our study of Wnt cleavage by Tiki has uncovered a new dimension of Wnt regulation that depends on unsuspected but critical Wnt biogenic properties. As Wnt signaling plays essential roles in diseases including cancer and osteoporosis, TIKI1/2 as likely Wnt-proteolytic enzymes may represent new therapeutic targets.

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EXPERIMENTAL PROCEDURES

Xenopus embryo manipulations and cDNA library screening

Procedures for embryo manipulation, reverse transcription PCR and *in situ* hybridization were performed as previously described (Tamai et al., 2000). The Arrayed *Xenopus tropicalis* full length cDNAs (Voigt et al., 2005) were used for the screening as performed previously (Kato et al., 2002). cDNA pools (in the CS107 vector) that contain 96 independent clones were transcribed in vitro using SP6 polymerase, and the resulting mRNA pools were injected into the dorsal side of the embryo at 4-cell stage at the dose of 10 ng RNA/embryo. Injected embryos were scored at stage 30-31 for enlarged or diminished anterior development. The positive pools were divided into smaller pools and the same process was repeated till a single cDNA was identified.

Morpholino injection

To examine Tiki1MO specificity, 500 pg Tiki1-FLAG (targeted by MO) or HA*Tiki1 (resistant to MO) were injected with or without the Tiki1MO (5, 10, 20, 40 ng) into the animal pole at the 2-cell stage, and animal caps were dissected at stage 10 for Western blotting. To knockdown the endogenous Tiki1, 20 ng of control MO, Tiki1MO or Tiki1MO together with 50 pg of HA-Tiki1 mRNA was injected into two dorsal blastomeres at the 8-cell stage and the phenotype was scored at stage 42.

Cell culture, transfection and reporter assay

HEK293T, HeLa, L and L-Wnt3a cells (from ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-glutamine (Invitrogen). Lipofectamine 2000 (Invitrogen) or Fugene6 (Roche) was used for all cell transfections. L and L-Wnt3a cells that stably express TIKI2 were generated by infecting cells with retroviruses and selected by puromycin. L cells stably expressing Wnt3a(FLAG) were established by transfecting L cells with a Wnt3a(FLAG) expressing vector and selected with neomycine. L cells stably expressing both Wnt3a(FLAG) and TIKI2 were also generated. TOPFLASH reporter assay was performed as previously described (MacDonald et al., 2008).

RNAi

To knockdown human TIKI2 in the TOPFLASH reporter assay, HEK293T cells in 24-well plates were transfected with 100 ng of TOPFLASH reporter, 5 ng of TK-Renilla reporter, 5 ng of empty vector or Wnt3a, and 20 nM of each siRNA. The reporter activities were examined 48 hrs post-transfection. To examine the cleavage of Wnt3a by endogenous TIKI2, 150 ng of HA-Wnt3a was transfected together with 20 nM of each siRNA into HEK293T cells in a 12-well plate, and after 48 hrs, the resulted CM were analyzed by Western blotting with anti-HA and anti-Wnt3a antibodies.

In vitro treatment of Wnt3a with purified proteins

About 50 ng purified TIKI2N, KRM2N, or TIKI2NΔ protein was mixed with 10 ng purified HA-Wnt3a in a total volume of 10 μl HEPES buffer (50 mM HEPES (pH 7.4), 100 mM NaCl, 0.2% NP-40) and incubated at 30° for 12 hrs. To examine TIKI2N activity in different pH conditions, the following buffer was used instead of HEPES, 100 mM sodium acetate (pH 5.5), 100 mM Tris-HCl (pH 6, 7, 8, 9) or 50mM CAPS (pH 10, 11), and the reaction was incubated for 4 hrs. To examine the effect of inhibitors on TIKI2N activity, the inhibitors (dissolved in DMSO) were added in the reaction system for 4 hrs and DMSO was used as a negative control. To examine the effect of divalent metals, 50 μM of each divalent metals ion was applied in the *in vitro* reaction and incubated for 2 hrs. To rescue TIKI2N

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activity with divalent metals after inhibition with 1, 10-Phenanthroline, 20 μM of 1, 10-Phenanthroline was firstly applied in the in vitro reaction for 1 hr and 50 μM of each metal ion was added and incubated for another 2 hrs. The reaction product was analyzed by SDS-PAGE and Western blotting with anti-HA and anti-Wnt3a antibodies. For mass spectrometric analysis, 200 ng of recombinant Wnt3a (from Stem RD) was incubated with about 500 ng of TIKIN or KRM2N overnight at 30° and separated by SDS-PAGE. The Wnt3a bands were cut, trypsin digested and subjected to mass spectrometric analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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REFERENCES

Banziger C, Soldini D, Schutt C, Zipperlen P, Hausmann G, Basler K. Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells. Cell. 2006; 125:509–522. [PubMed: 16678095]

Barrett, AJ.; Rawlings, ND.; Woessner, JF. Handbook of Proteolytic Enzymes. Academic Press; San Diego: 1998.

Brannon M, Gomperts M, Sumoy L, Moon RT, Kimelman D. A beta-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in Xenopus. Genes & development. 1997; 11:2359–2370. [PubMed: 9308964]

Cho KW, Blumberg B, Steinbeisser H, De Robertis EM. Molecular nature of Spemann's organizer: the role of the Xenopus homeobox gene goosecoid. Cell. 1991; 67:1111–1120. [PubMed: 1684739]

Christian JL, Moon RT. Interactions between Xwnt-8 and Spemann organizer signaling pathways generate dorsoventral pattern in the embryonic mesoderm of Xenopus. Genes & development. 1993; 7:13–28. [PubMed: 8422982]

Clevers H. Wnt/beta-catenin signaling in development and disease. Cell. 2006; 127:469–480. [PubMed: 17081971]

Cong F, Schweizer L, Varmus H. Wnt signals across the plasma membrane to activate the beta-catenin pathway by forming oligomers containing its receptors, Frizzled and LRP. Development (Cambridge, England). 2004; 131:5103–5115.

Coombs GS, Yu J, Canning CA, Veltri CA, Covey TM, Cheong JK, Utomo V, Banerjee N, Zhang ZH, Jadulco RC, et al. WLS-dependent secretion of WNT3A requires Ser209 acylation and vacuolar acidification. Journal of cell science. 2010; 123:3357–3367. [PubMed: 20826466]

De Robertis EM, Kuroda H. Dorsal-ventral patterning and neural induction in Xenopus embryos. Annual review of cell and developmental biology. 2004; 20:285–308.

Franch-Marro X, Wendler F, Griffith J, Maurice MM, Vincent JP. In vivo role of lipid adducts on Wingless. Journal of cell science. 2008; 121:1587–1592. [PubMed: 18430784]

Harland R, Gerhart J. Formation and function of Spemann's organizer. Annual review of cell and developmental biology. 1997; 13:611–667.

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Hausmann G, Banziger C, Basler K. Helping Wingless take flight: how WNT proteins are secreted. Nature reviews. 2007; 8:331–336.

He X, Semenov M, Tamai K, Zeng X. LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. Development (Cambridge, England). 2004; 131:1663–1677.

Kato Y, Habas R, Katsuyama Y, Naar AM, He X. A component of the ARC/Mediator complex required for TGF beta/Nodal signalling. Nature. 2002; 418:641–646. [PubMed: 12167862]

Komekado H, Yamamoto H, Chiba T, Kikuchi A. Glycosylation and palmitoylation of Wnt-3a are coupled to produce an active form of Wnt-3a. Genes Cells. 2007; 12:521–534. [PubMed: 17397399]

Kurayoshi M, Yamamoto H, Izumi S, Kikuchi A. Post-translational palmitoylation and glycosylation of Wnt-5a are necessary for its signalling. Biochem J. 2007; 402:515–523. [PubMed: 17117926]

Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annual review of cell and developmental biology. 2004; 20:781–810.

MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell. 2009; 17:9–26. [PubMed: 19619488]

MacDonald BT, Yokota C, Tamai K, Zeng X, He X. Wnt signal amplification via activity, cooperativity, and regulation of multiple intracellular PPPSP motifs in the Wnt co-receptor LRP6. The Journal of biological chemistry. 2008; 283:16115–16123. [PubMed: 18362152]

Mao B, Wu W, Davidson G, Marhold J, Li M, Mechler BM, Delius H, Hoppe D, Stannek P, Walter C, et al. Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. Nature. 2002; 417:664–667. [PubMed: 12050670]

Molenaar M, van de Wetering M, Oosterwegel M, Peterson-Maduro J, Godsave S, Korinek V, Roose J, Destree O, Clevers H. XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. Cell. 1996; 86:391–399. [PubMed: 8756721]

Moon RT, Kohn AD, De Ferrari GV, Kaykas A. WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet. 2004; 5:691–701. [PubMed: 15372092]

Mulligan KA, Fuerer C, Ching W, Fish M, Willert K, Nusse R. Secreted Wingless-interacting molecule (Swim) promotes long-range signaling by maintaining Wingless solubility. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109:370–377. [PubMed: 22203956]

Niehrs C. Regionally specific induction by the Spemann-Mangold organizer. Nat Rev Genet. 2004; 5:425–434. [PubMed: 15153995]

Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics. 2002; 1:376–386. [PubMed: 12118079]

Overall CM, Blobel CP. In search of partners: linking extracellular proteases to substrates. Nature reviews. 2007; 8:245–257.

Petersen CP, Reddien PW. Wnt signaling and the polarity of the primary body axis. Cell. 2009; 139:1056–1068. [PubMed: 20005801]

Singh S, Springer M, Steen J, Kirschner MW, Steen H. FLEXIQuant: a novel tool for the absolute quantification of proteins, and the simultaneous identification and quantification of potentially modified peptides. Journal of proteome research. 2009; 8:2201–2210. [PubMed: 19344176]

Strigini M, Cohen SM. Wingless gradient formation in the Drosophila wing. Curr Biol. 2000; 10:293–300. [PubMed: 10744972]

Takada R, Satomi Y, Kurata T, Ueno N, Norioka S, Kondoh H, Takao T, Takada S. Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion. Dev Cell. 2006; 11:791–801. [PubMed: 17141155]

Tamai K, Semenov M, Kato Y, Spokony R, Liu C, Katsuyama Y, Hess F, Saint-Jeannet JP, He X. LDL-receptor-related proteins in Wnt signal transduction. Nature. 2000; 407:530–535. [PubMed: 11029007]

Tamai K, Zeng X, Liu C, Zhang X, Harada Y, Chang Z, He X. A mechanism for Wnt coreceptor activation. Mol Cell. 2004; 13:149–156. [PubMed: 14731402]

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Voigt J, Chen JA, Gilchrist M, Amaya E, Papalopulu N. Expression cloning screening of a unique and full-length set of cDNA clones is an efficient method for identifying genes involved in Xenopus neurogenesis. Mechanisms of development. 2005; 122:289–306. [PubMed: 15763209]

Wei Q, Yokota C, Semenov MV, Doble B, Woodgett J, He X. R-spondin1 is a high affinity ligand for LRP6 and induces LRP6 phosphorylation and beta-catenin signaling. The Journal of biological chemistry. 2007; 282:15903–15911. [PubMed: 17400545]

Willert K, Brown JD, Danenberg E, Duncan AW, Weissman IL, Reya T, Yates JR 3rd, Nusse R. Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature. 2003; 423:448–452. [PubMed: 12717451]

Zecca M, Basler K, Struhl G. Direct and long-range action of a wingless morphogen gradient. Cell. 1996; 87:833–844. [PubMed: 8945511]

Zhai L, Chaturvedi D, Cumberledge S. Drosophila wnt-1 undergoes a hydrophobic modification and is targeted to lipid rafts, a process that requires porcupine. The Journal of biological chemistry. 2004; 279:33220–33227. [PubMed: 15166250]

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Research Highlights

- Tiki1 is expressed in Spemann Organizer and is required for head formation in *Xenopus*
- Tiki1 inactivates Wnt proteins via cleaving Wnt amino terminus
- Tiki1 acts in both Wnt-producing and Wnt-responding cells
- Wnt amino terminus and palmitoylation act to minimize Wnt oxidation-inactivation

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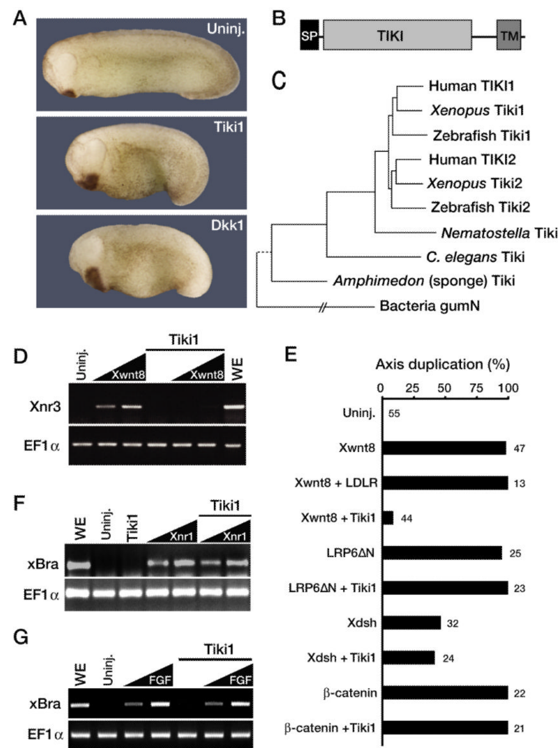

Figure 1. Tiki1 promotes anterior development and inhibits Wnt signaling

(A) Dorsal injection of Tiki1 mRNA (100 pg) induced enlarged head similar to that by Dkk1.

(B) Tiki1 domain scheme. SP, signal peptide; TM, transmembrane.

(C) Phylogenetic tree of Tiki proteins from human, *Xenopus*, zebrafish, *Nematostella*, C.*elegans* and *Amphimedon* by ClustalW (see Figure S1). TIKI domain is homologous to bacterial gumN (see Figure S2A).

(D) Tiki1 inhibited Xwnt8-induced *Xnr3* expression in animal caps. EF-1α: a loading control. uninj: uninjected embryos; WE: whole embryos.

(E) Tiki1 inhibited axis duplication by Xwnt8 but not by LRP6ΔN, Xdsh or β-catenin. LDLR: a control. Numbers indicate embryos scored. (F and G) Tiki1 did not inhibit Nodal or FGF signaling.

See also Figures S1 and S2.

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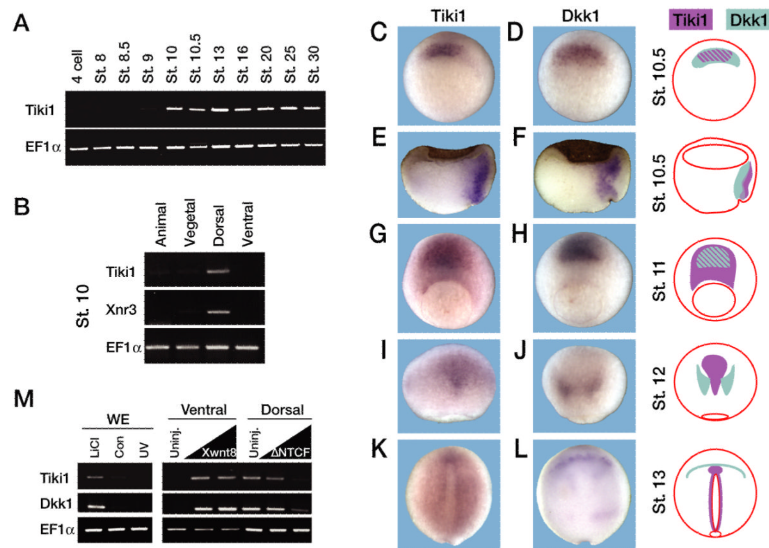

Figure 2. *Tiki1* expression and regulation in early embryos

(A) RT-PCR revealed that *Tiki1* mRNA is detectable from stage 9 to 30.

(B) *Tiki1* is expressed dorsally. Embryos were cut into animal, vegetal, dorsal, and ventral parts for RT-PCR. *Xnr3*: a positive control.

(C-L) Whole mount *in situ* hybridization of *Tiki1* and *Dkk1* during gastrulation. Drawing is on right. C and D, vegetal view, dorsal on top; E and F, hemi-section, dorsal on right; G to L, dorsal view, anterior on top.

(M) Tiki1 expression depends on maternal Wnt signaling. Con: control, UV: ultraviolet.

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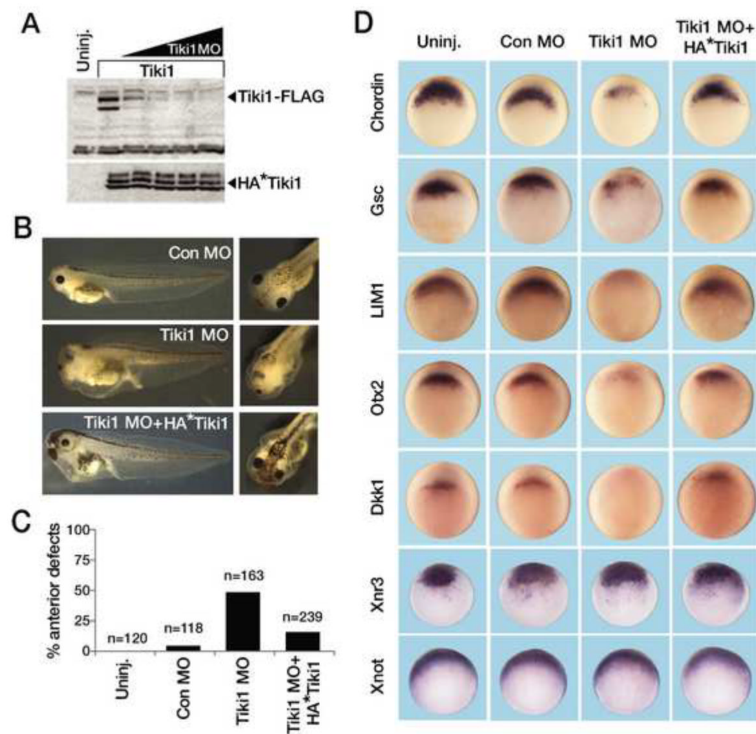

Figure 3. Tiki1 is required for anterior development

(A) Tiki1MO (5, 10, 20, and 40 ng) inhibited protein expression from Tiki1-FLAG but not HA*Tiki1 mRNAs (500 pg).

(B) Tiki1MO (20 ng) caused anterior defects, which were rescued by HA*Tiki1 mRNA (50 pg). Lateral (left) and dorso-anterior (right) views are shown at stage 42.

(C) Statistical data of the Tiki1MO phenotype.

(D) Tiki1MO suppressed head organizer gene expression at stage 10.5-11. See also Figure S3 and Tables S1-3.

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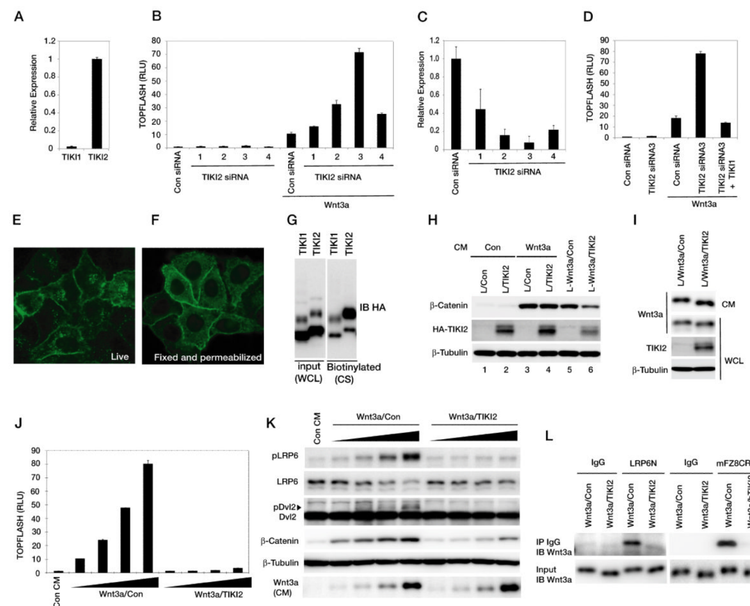

Figure 4. Tiki inactivates Wnt3a

(A) Relative expression levels of TIKI1 and TIKI2 mRNAs in HEK293T cells.

(B) Knocking down endogenous TIKI2 in HEK293T cells enhanced Wnt3a signaling.

(C) TIKI2 mRNA knockdown examined 48 hrs after siRNA transfection.

(D) The TIKI2 knockdown effect was countered by TIKI1 expression.

(E, F) Immunofluorescence of HA*Tiki1 expressed in HeLa cells. Live cells (E) or fixed-permeabilized cells (F) were labeled by an anti-HA antibody and a fluorescent secondary antibody.

(G) HeLa cells expressing HA-TIKI1 or HA-TIKI2 were treated with a non-permeable biotinylation reagent. Cell surface (CS) proteins were precipitated with streptavidin beads and blotted for HA-TIKI1/2. WCL: input whole cell lysates. The slower migrating form of HA-TIKI, due possibly to glycosylation, was enriched on the cell surface.

(H) TIKI2 inhibited β-catenin stabilization in Wnt3a-expressing cells. Indicated L cell lines were also treated with control or Wnt3a CM for 2 hrs (lanes 1-4). β-tubulin: a loading control.

(I) Wnt3a was secreted similarly with or without TIKI2, but exhibited faster migration due to TIKI2.

(J) Wnt3a CM from TIKI2-expressing L cells exhibited minimal activity, tested at concentrations in 2-fold dilutions.

(K) Wnt3a CM from TIKI2-expressing L cells induced minimal LRP6 or Dvl2 phosphorylation or β-catenin stabilization at 2 hours. LRP6 intensity reduction was not due to decreased protein levels but due to LRP6 phosphorylation, which perturbed recognition by the LRP6 antibody used (not shown).

(L) Wnt3a secreted from TIKI2-expressing cells exhibited minimal binding to mFz8CRD-IgG or LRP6N-IgG (Tamai et al., 2000).

See also Figure S4.

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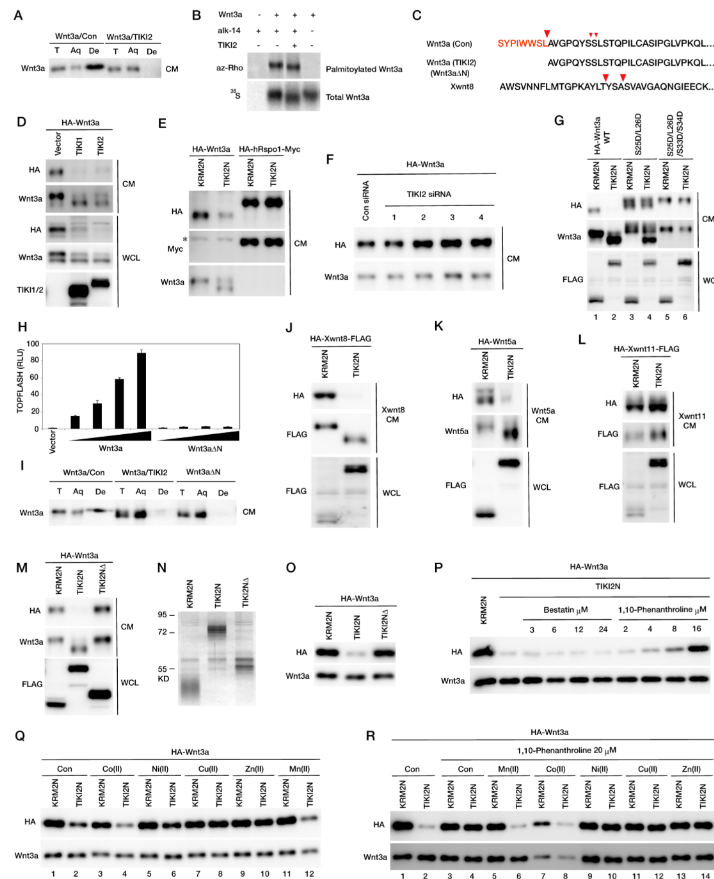

Figure 5. Tiki inactivates Wnt3a via amino-terminal cleavage

(A) Triton X-114 phase separation. Control Wnt3a partitioned in detergent (De), but Wnt3a from TIKI2-expressing cells partitioned in aqueous (Aq) phase. T, total.

(B) TIKI2 did not affect Wnt3a acylation (C77 plus S209) in metabolic labeling.

(C) Amino-terminal cleavage of Wnt3a and Xwnt8 by TIKI2. The big and small arrowheads indicate major and minor cleavage sites, respectively (see Figure S5). Wnt3aΔN was engineered to be identical to TIKI-cleaved Wnt3a.

(D) TIKI cleaved the HA tag from HA-Wnt3a. TIKI1 cDNA was transfected at a 10-fold higher dose than that of TIKI2 for similar protein levels.

(E) TIKI did not cleave Rspo1. * non-specific.

(F) Endogenous TIKI2 cleaved Wnt3a in HEK293T cells. TIKI2 siRNAs were as in Figures 4B and 4C.

(G) Wnt3a(S25D/L26D) and Wnt3a(S25D/L26D/S33D/S34D) were partially and fully resistant to TIKI2N cleavage, respectively (lanes 4 and 6 versus 2). Both mutants exhibited slower mobility.

(H) Wnt3aΔN was inactive as examined by cDNA transfection in 2-fold dilutions.

(I) Wnt3aΔN in CM partitioned in the aqueous phase.

(J-L) TIKI2 cleaved the HA tag from HA-Xwnt8-FLAG and HA-Wnt5a, but not HA-Xwnt11-FLAG. Levels of KRM2N and TIKI2N (both FLAG-tagged) were monitored.

(M) HA-Wnt3a was cleaved by TIKI2N, but not by KRM2N or TIKI2NΔ FLAG-tagged), in HEK293T cells.

(N) Silver staining of purified KRM2N, TIKI2N or TIKI2NΔ. (O) Purified TIKI2N, but neither KRM2N nor TIKI2NΔ, cleaved recombinant HA-Wnt3a *in vitro*.

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(P) TIKI2N cleavage of HA-Wnt3a was inhibited by 1, 10-Phenanthroline but not Bestatin. 16 μM of 1, 10-Phenanthroline is about 100 times lower than manufacturer's recommended doses.

(Q) TIKI2N cleavage of HA-Wnt3a was enhanced by Co^{2+} or Mn^{2+}, but inhibited by Ni^{2+}, Cu^{2+}, or Zn^{2+}.

(R) TIKI2N inhibition by 1, 10-Phenanthroline was rescued by Co^{2+} or Mn^{2+} (lanes 6 and 8).

See also Figures S4, S5, S6, and S7.

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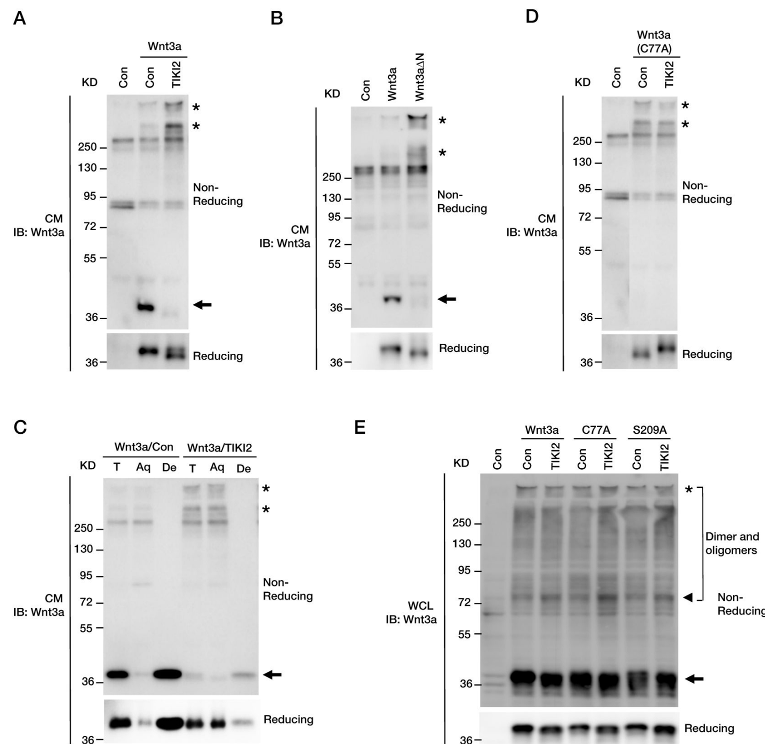

Figure 6. Wnt3a oxidation-oligomerization due to Tiki cleavage

(A-D) Secreted Wnt3a in CM. The arrow and * indicate Wnt3a monomers and oligomers in non-reducing gels, respectively (top). Note the enormous size of the upper * band. Reducing gels (bottom) were also shown.

(A) Wnt3a from TIKI2-expressing cells formed oxidized oligomers exclusively.

(B) Wnt3aΔN formed oxidized oligomers.

(C) Oxidized Wnt3a oligomers partitioned exclusively in the aqueous phase, while Wnt3a monomers partitioned in detergent.

(D) Wnt3a(C77A) formed oxidized oligomers regardless of TIKI2. This experiment was performed together with the one presented in (A) and a common control lane was used. Secreted Wnt3a(C77A) after TIKI2 cleavage migrated slower in the reducing gel due to altered N-glycosylation (see Figure S4E).

(E) Wnt3a, Wnt3a(C77A), and Wnt3a(S209A) behaved similarly in whole cell lysates. Each existed as monomers (arrow) and heterogeneous oxidized species from dimers (arrowhead) to larger oligomers (*), regardless of TIKI2 cleavage.

See also Figures S6 and S7.

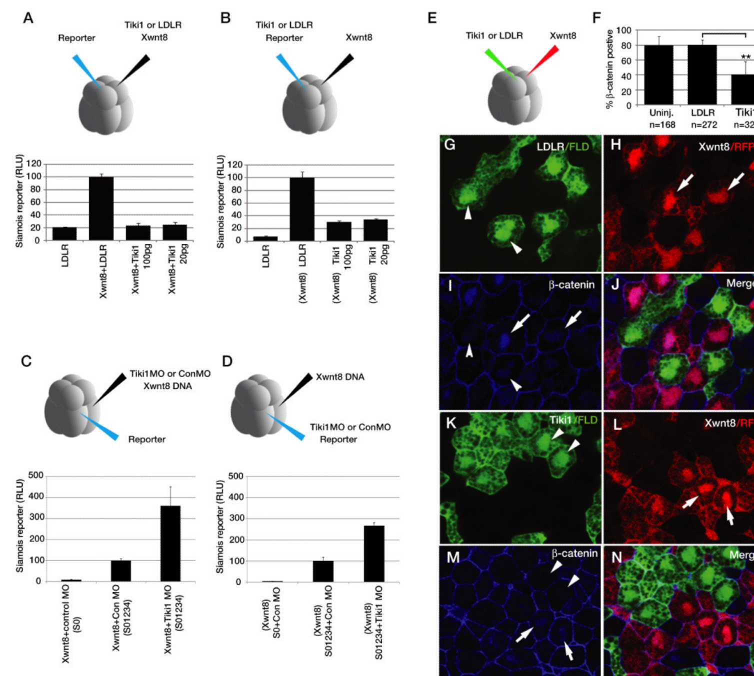

Figure 7. Tiki1 functions in both Wnt-producing and Wnt-responding cells

(A) Tiki1 acts in Xwnt8-producing cells. Tiki1 mRNA (100 pg) co-injected with Xwnt8 mRNA (20 pg) inhibited Xwnt8-induced S01234-Luciferase in neighboring cells. LDLR: a control.

(B) Tiki1 acts in Xwnt8-responding cells. Tiki1 mRNA co-injected with S01234-Luciferase inhibited the reporter induced by Xwnt8 from neighboring cells that received Xwnt8 mRNA.

(C) The endogenous Tiki1 acts in Wnt-producing cells. Tiki1MO (20 ng) co-injected with CS2+Xwnt8 DNA (40 pg) in a single dorsal blastomere enhanced Xwnt8-induced S01234-Luciferase in neighboring cells. ConMO: control MO.

(D) The endogenous Tiki1 acts in Wnt-responding cells. Tiki1MO co-injected with S01234-Luciferase enhanced the reporter induced by Xwnt8 from neighboring cells that received CS2+Xwnt8 DNA.

(E-N) Tiki1 reduces nuclear β-catenin levels by acting in Wnt-responding cells. Tiki1 or LDLR mRNA (200pg) (FLD^+, green) and Xwnt8 mRNA (20 pg) (RFP^+, red) were injected into neighboring blastomeres, respectively. Stage 9 animal cap cells were imaged with an anti-β-catenin antibody (blue). Examples of Xwnt8-expressing cells (arrows) and Tiki1- or LDLR-expressing cells (arrowheads) are highlighted. Only cells within the distance of five cell bodies from Xwnt8-expressing cells were counted. No nuclear β-catenin-positive cells were found in areas free of Xwnt8-expressing cells (not shown). All cells were positive for plasma membrane-associated β-catenin. Statistical data were derived from three independent experiments (F). **$p<0.001$.

See also Figures S7.

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