A Chemical and Genetic Approach to the Mode of Action of Fumagillin

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Summary

Previous mode of action studies identified methionine aminopeptidase 2 (MetAP-2) as the target of the antiangiogenic natural product fumagillin and its drug candidate analog, TNP-470. We report here that TNP-470-mediated MetAP-2 inhibition blocks noncanonical Wnt signaling, which plays a critical role in development, cell differentiation, and tumorigenesis. Consistent with this finding, antisense MetAP-2 morpholino oligonucleotide injection in zebrafish embryos phenocopies gastrulation defects seen in noncanonical Wnt5 loss-of-function zebrafish mutants. MetAP-2 inhibition or depletion blocks signaling downstream of the Wnt receptor Frizzled, but upstream of Calmodulin-dependent Kinase II, RhoA, and c-Jun N-terminal Kinase. Moreover, we demonstrate that TNP-470 does not block the canonical Wntβ-catenin pathway. Thus, TNP-470 selectively regulates noncanonical over canonical Wnt signaling and provides a unique means to explore and dissect the biological systems mediated by these pathways.

Introduction

Targeting the tumor vasculature by using antiangiogenic therapeutics has been an attractive strategy to limit the size and metastases of tumors. One of the most potent small-molecule antiangiogenic agents is the natural product fumagillin (1), which was isolated in the Folkman lab from a contaminating Aspergillus fumagatus colony in an endothelial cell culture. Fumagillin was subsequently chemically modified to create the drug candidate TNP-470 (2) [1], which was tested in Phase I/II trials for Kaposi’s sarcoma, renal cell carcinoma, brain cancer, breast cancer, cervical cancer, and prostate cancer [2]. Using a chemical genetics approach, we previously identified methionine aminopeptidase 2 (MetAP-2) as a direct binding protein of fumagillin [3], and we published the structure of human MetAP-2 bound to fumagillin [4]. Both fumagillin and TNP-470 specifically bind MetAP-2 and inhibit its activity [3, 5]. However, a role for MetAP-2 in an intracellular signaling pathway has not been identified. Here, we continue our mode of action studies of TNP-470 by combining model organism (zebrafish) small-molecule chemical genetics with nucleic acid-based genetic techniques (MO and siRNA) to identify TNP-470 as a selective inhibitor of noncanonical Wnt signaling.

How cells coordinate polarity and movement within a larger population is a fundamental question in development biology. Genetic studies of the Drosophila wing epithelia have identified several genes that function to establish asymmetric cell polarities by coordinating the organization of the cytoskeleton [6]. These genes comprise the planar cell polarity pathway (PCP), which includes some genes known to be components (i.e., Frizzled, Dishevelled) of the canonical Wnt signaling pathway that leads to β-catenin-mediated transcription (Figure 1). Additional core PCP proteins constituting a “noncanonical” Wnt signaling pathway include Daam1, RhoA, c-Jun N-terminal Kinase (JNK), and CaMKII. In addition to Drosophila wing epithelia polarization, noncanonical Wnt signaling has been implicated in the complex coordination of cell polarity, adhesion, and movement required for vertebrate gastrulation. A key set of morphogenetic movements during gastrulation is the mediolateral narrowing and anterior-posterior tissue lengthening necessary to form the embryonic axis [7]. Studies have demonstrated that these “convergence and extension” (CE) movements are regulated by noncanonical signaling [8, 9]. For example, Wnt5 has been implicated in the morphogenetic movements during Xenopus and zebrafish gastrulation [10–12]. Unlike canonical Wnt signaling, which is fairly well characterized, noncanonical Wnt signaling pathways are not fully elucidated. For example, it remains to be determined if multiple noncanonical pathways exist since various subsets of genes reported to be involved in noncanonical Wnt signaling have been linked to biological systems as diverse as neurogenesis [13], cell adhesion [14], and cell polarization [15, 16]. Alternatively, a “core” noncanonical Wnt pathway may exist, and these observed differences may simply reflect the differences inherent in the various systems studied. Unlike canonical Wnt signaling, for which both small-molecule agonists and antagonists have been reported [17, 18], the study of noncanonical Wnt signaling has been hampered by the lack of specific small-molecule probes of this pathway.

To follow up on our recent finding that MetAP-2 knockout murine embryos fail to undergo gastrulation [19], here we explore the role of MetAP-2 in early zebrafish development. We show that MetAP-2 is necessary for noncanonical Wnt signaling in vivo and in cell culture systems. Moreover, we report that the MetAP-2 inhibitor TNP-470 selectively and potently inhibits noncanonical Wnt signaling downstream or at the level of Frizzled receptors and upstream of JNK and CaMKII activation. These studies demonstrate the first small-molecule inhibitor for the investigation of the diverse biological systems mediated by these important signaling pathways.
Results

MetAP-2 Genetically Interacts with Wnt5 in Zebrafish

Using the zebrafish (Danio rerio) developmental process as a chemical genetic model to elucidate the mode of action of the small-molecule compound TNP-470, we treated zebrafish embryos with the MetAP-2 inhibitor fumagillin (1) and TNP-470 (2) (Figure 2). While these experiments resulted in a truncated tail phenotype (data not shown), the penetrance was low, possibly due to poor permeability across the chorionic membrane. We identified and cloned zebrafish MetAP-2 (chromosome 25), and it has ubiquitous expression in early development. Interestingly, at the time of vascularization, this MetAP-2 transcript is enriched in the intersomitic regions (Figure S1; see the Supplemental Data available with this article online). Thus, we next knocked down MetAP-2 expression in zebrafish embryos by antisense morpholino oligonucleotides (MO) injection. The resulting zebrafish embryos displayed a highly penetrant truncated posterior phenotype similar to the AP axis tail extension defect observed in Wnt5 MO-injected zebrafish, although both of these low-dose MOs show milder gastrulation defects than the Wnt5 mutant pipetail (ppt) (Figures 3A–3D). This tail phenotype was also observed by using a second MO targeting a different splice site not observed in 5 base pair mismatch MetAP-2 MO-injected embryos (data not shown). Downregulation of MetAP-2 protein was confirmed by immunoblot analysis (Figure S2). Interestingly, embryos coinjected with a combination of low-dose MetAP-2 and Wnt5 MOs resulted in a strong synergistic effect rather than an additive effect, suggesting a genetic interaction between these two molecules (Figure 3E; Table 1). Furthermore, these MetAP-2 MO-injected zebrafish displayed broader MyoD somite staining consistent with the observed MyoD staining in ppt fish [20] (Figure S3). This gastrulation defect is similar to but not as severe as the complete loss of gastrulation in the murine MetAP-2 knockout mouse [19]. This discrepancy between the two model systems may be due to (1) an incomplete antisense MO MetAP-2 knockdown in the zebrafish system, (2) the significant contribution of pre-synthesized maternal MetAP-2 protein in the fish egg, which could partially rescue loss of embryonic MetAP-2 mRNA, or (3) genetic redundancy as a result of the teleost genomic duplication (we recently identified a related MetAP-2 gene on chromosome 4).

To evaluate if MetAP-2 has a general role in noncanonical signaling or is only specific for Wnt5 signaling, zebrafish embryos were coinjected with MOs targeting MetAP-2 and the noncanonical Wnt11. Injection of the Wnt11 MO alone resulted in 12% of the embryos displaying a moderate or severe eye defect consistent with the phenotype of the Wnt11 mutant silberblick (slb). However, coinjection with MetAP-2 MO more than tripled (38%) the observed moderate/severe eye phenotype (Table S1).

It has been shown that Wnt5a activates noncanonical Wnt signaling through intracellular Ca\(^{2+}\) release [21] and the activation of Ca\(^{2+}\)-sensing enzymes such as CamKII. In addition, it has been demonstrated that injection of truncated, constitutively activated CamKII (CamKIItr) mRNA is sufficient to rescue the Wnt5 homozygous mutant tail defect [22]. Thus, we wanted to determine if exogenous CamKII activity was also able to suppress the MetAP-2 MO-induced phenotypes. As demonstrated in a genetic rescue experiment (Figure 3F; Table 1),
coinjection of CamKIItr mRNA with MetAP-2 MO partially suppressed the MO-induced phenotype (from a 37% frequency of shortened AP axis in MetAP-2 MO only to a 17% frequency in MetAP-2 MO plus CamKIItr). Thus, these genetic experiments clearly demonstrate a requirement for MetAP-2 in noncanonical Wnt5 signaling.

**MetAP-2 Is Required for Noncanonical, but Not Canonical, Wnt Signaling**

Since the mouse teratocarcinoma F9 system played an important role in elucidating the noncanonical Wnt5a/Rfz2 pathway in mammalian cells and faithfully recapitulates what is observed in zebrafish [23, 24], we chose to utilize this system to confirm the genetic interaction between MetAP-2 and Wnt5a observed in zebrafish embryos. In this assay, the activation of either canonical or noncanonical Wnt receptors (e.g., Fz1 and Fz2, respectively) leads to differentiation into primitive endoderm (PE), as measured by the induction of several markers such as tissue plasminogen activator (tPA) and the cytokeratin Endo-A[23, 24]. We therefore tested the ability of TNP-470 (2) to disrupt Wnt5a-mediated induction of PE in the F9 cell system. As shown in Figure 4A, 10 nM TNP-470 was sufficient to inhibit PE induction, as assessed by tPA activity levels in F9 cells expressing the noncanonical Wnt receptor Fz2 when cocultured with Wnt5a-expressing HEK293 cells. In contrast, PE induction by retinoic acid was unaffected. This inhibition was not due to the cytostatic activity of TNP-470 observed in endothelial cells since 10 nM TNP-470 had a negligible effect on F9 cell proliferation (Figure S4). In addition, we found that directly knocking down 90% of MetAP-2 expression with MetAP-2 gene (MAP2)-specific siRNA resulted in a dramatic decrease in PE induction to levels similar to those seen in TNP-470-treated cells (Figure 4B). In parallel experiments in which cytokeratin Endo-A antibody (TROMA-1) staining was used to monitor PE induction, TNP-470 potently inhibited Wnt5a/Fz2 signaling (Figure 4C). Similarly, siRNA-mediated MetAP-2 downregulation also blocked Wnt5a-induced cytokeratin Endo-A induction (Figure 3C). These data indicate that MetAP-2 activity is required for Wnt5a function.

**Table 1. Wnt5 and MetAP-2 Interact Genetically**

<table>
<thead>
<tr>
<th></th>
<th>% Wild-Type</th>
<th>% Moderately Morphant</th>
<th>% Highly Morphant</th>
<th>% Severely Truncated</th>
<th>n</th>
</tr>
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<tr>
<td>150 μM Wnt5 and 300 μM MetAP-2 MO</td>
<td>1.8</td>
<td>54.1</td>
<td>31.2</td>
<td>12.9</td>
<td>109</td>
</tr>
<tr>
<td>150 μM Wnt5 MO</td>
<td>89.3</td>
<td>8.0</td>
<td>2.7</td>
<td>0</td>
<td>75</td>
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<tr>
<td>300 μM MetAP-2 MO</td>
<td>80.6</td>
<td>12.2</td>
<td>6.1</td>
<td>1.1</td>
<td>98</td>
</tr>
<tr>
<td>600 μM MetAP-2 MO plus control</td>
<td>27</td>
<td>35</td>
<td>37</td>
<td>0</td>
<td>137</td>
</tr>
<tr>
<td>600 μM MetAP-2 MO plus CamKIItr</td>
<td>53</td>
<td>30</td>
<td>17</td>
<td>0</td>
<td>182</td>
</tr>
</tbody>
</table>

Wnt5 (150 μM) and MetAP-2 (300 μM) antisense morpholinos act synergistically to elicit tail extension defects, and activated CamKIItr partially rescues the MetAP-2 MO phenotype. (“Moderately Morphant” describes a weaker ppt mutant phenotype, “Highly Morphant” embryos have a strong ppt phenotype, and “Severely Truncated” describes embryos that fail to extend past the yolk.)
While generally believed to be a noncanonical Wnt family member [25], Wnt5a has also been reported to induce axis duplication in Xenopus embryos when overexpressed with the canonical Frizzled Fz5 [26]. Since both canonical and noncanonical Wnt signaling can induce PE formation in the F9 cell system [23, 24], we investigated whether both pathways require MetAP-2. To avoid any ambiguity regarding crosstalk between Wnt5a and different Frizzled receptors, we used isoproterenol-responsive chimeric Frizzled receptors, which have proven useful in the study of signaling events downstream of canonical and noncanonical Frizzled proteins [27, 28]. These chimeric receptors consist of the extracellular and transmembrane domains of β2-adrenergic receptor (β2-AR) and the putative intracellular loops of the rat Frizzled-1/Rfz1 (canonical) or Frizzled-2/Rfz2 (noncanonical) receptors. Activation with the β2-AR agonist isoproterenol of either chimeric receptor individually expressed in F9 cells leads to PE induction (Figure 5A).

Confirming the observed TNP-470 inhibition of Wnt5a-induced PE formation (Figures 4A and 4C), we observed that isoproterenol-induced PE formation through β2-AR/Rfz2 in F9 cells was attenuated by TNP-470, as assayed by TROMA-1 staining. Similarly, knockdown of endogenous MetAP-2 by siRNA blocked isoproterenol-triggered PE formation in F9 cells expressing β2-AR/Rfz2 (Figure 5A). In contrast, PE induction through β2-AR/Rfz1 was not affected by TNP-470 or by siRNA targeting MAP2 (Figure 5A). Thus, our results strongly suggest that MetAP-2 is required for signaling through the noncanonical Wnt receptor Frizzled 2, but not the canonical Wnt receptor Frizzled 1.

It is well established that activation of the canonical Wnt pathway leads to β-catenin stabilization and nuclear translocation, which subsequently activates lymphoid-enhancer factor (LEF)/T cell factor (TCF)-regulated transcription [29, 30]. To confirm that MetAP-2 function is not involved in the canonical Wnt pathway, we tested whether fumagillin (1), the potent parent compound of TNP-470, regulates LEF/TCF transactivation by using the LEF/β-catenin-responsive TOPflash-luciferase reporter [31]. As shown in Figure 5C, 100 μM isoproterenol induced a 6-fold increase in TCF-luciferase after 6 hr in β2-AR/Rfz1 cells. Preincubation with 10 nM fumagillin had no effect on TCF transactivation. Thus, once again, our results indicate that MetAP-2 activity does not affect the Wnt/β-catenin pathway.

MetAP-2 Acts Upstream of CaMKII, RhoA, and JNK Activation

To study further whether MetAP-2 is required for the induction of intracellular calcium, as suggested by our zebrafish experiments, we investigated the effect of MetAP-2 inhibition on CamKII in F9 cells. Treatment of β2-AR/Rfz2-expressing F9 cells with 100 μM isoproterenol for 10 min increased CamKII activity 4-fold (Figure 6A). However, this increase in CamKII activity was inhibited in cells pretreated with 10 nM TNP-470. Attenuation of MetAP-2 protein levels via siRNA also inhibited CamKII activation upon treatment with isoproterenol.
These data are consistent with the activated CamKIItr rescue of MetAP-2 downregulation in zebrafish (Figure 3F) and confirm the requirement of MetAP-2 activity in the Wnt/Ca2+ signaling pathway. Since JNK is reported to be downstream of noncanonical Wnt signaling [32, 33] and has been implicated in controlling endothelial cell proliferation [34], we next tested whether JNK activity can be regulated by TNP-470 by using phospho-63-c-jun antibody. As shown in Figure 6B, Wnt5a-stimulated JNK activity in Rfz2-expressing F9 cells was inhibited when these cells were pretreated with TNP-470 for 16 hr. This result indicates that MetAP-2 acts upstream of c-Jun activation.

The small GTPase RhoA is another important downstream component of the noncanonical Wnt signaling pathway. In pull-down assays with the Rho binding domain (RBD) from the effector protein Rhoetkin, Wnt5a-stimulated RhoA activity was blocked by pretreatment of mouse pulmonary endothelial (MPE) cells with TNP-470 for 16 hr (Figure 6C). It is interesting to note that both c-jun and RhoA are critical factors involved in angiogenesis [34, 35].

**Activation of Noncanonical Wnt Signaling Rescues TNP-470 Sensitivity in Endothelial Cells**

Given our result that MetAP-2 acts downstream of noncanonical Wnt signaling, we next explored whether TNP-470-induced cell cycle arrest in endothelial cells could be rescued by activation of the downstream Wnt effector Dishevelled. We used ΔDIX-Dvl2 that contained an amino-terminal deletion of the DIX domain of Dishevelled-2, which preferentially activated noncanonical Wnt signaling in a Fz-independent manner [8, 36]. Stable introduction of a ΔDIX-Dvl2 construct into MPE cells reduces endothelial cell sensitivity to TNP-470 by ~10-fold (Figure 6D). This was also observed in another endothelial cell type, HUVE cells stably expressing ΔDIX-Dvl2 (data not shown). These pooled retrovirus-infected HUVE cells do not represent an individual clone, thereby precluding the possibility that the distinct sensitivities are due to clonal differences in responsiveness. These data indicate that overexpression of activated Dishevelled-2 partially rescues the loss of noncanonical Wnt signaling upon TNP-470 treatment. In addition, these results suggest that inhibition of noncanonical Wnt signaling by TNP-470 may be partially responsible for its cytostatic effect. Taken together, our findings place the need for MetAP-2 activity in noncanonical Wnt signaling proximal to Frizzled activation of Dishevelled and upstream of calcium signaling, RhoA, and JNK activation [36].

**Discussion**

The identification of selective small-molecule inhibitors can greatly aid the investigation of intracellular signaling pathways. Here, we continue our mode of action studies of TNP-470 by combining model organism chemical genetic and traditional genetics approaches to demonstrate that the target of TNP-470, MetAP-2, is essential for noncanonical Wnt signaling, which plays a critical role in development, cell differentiation, and tumorigenesis. Given the several shared components between noncanonical and canonical Wnt/β-catenin signaling pathways, our finding that TNP-470 selectively inhibits noncanonical Wnt signaling over canonical Wnt signaling in a MetAP-2-dependent manner will aid the...
investigation of the diverse biological systems mediated by these important signaling pathways.

Our studies narrow the point at which MetAP-2 function is required in this signaling cascade. First, we demonstrated that MetAP-2 is downstream of Wnt and Fz, since noncanonical Wnt signaling initiated by the isoproterenol-inducible chimeric Fz2 receptor was inhibited by loss of MetAP-2 activity. While downstream of Fz, our data show that MetAP-2 is upstream of JNK and CaMKII activation. We then investigated signaling more proximal to Fz, and we showed that DIX-Dvl was able to convey partial resistance to TNP-470-mediated cell cycle arrest, suggesting that MetAP-2 function is required upstream or at the level of Dishevelled. Our finding that TNP-470 inhibits noncanonical Wnt signaling between Frizzled receptors and Dishevelled further highlights the fact that this is a critical step at which canonical and noncanonical Wnt pathways diverge and is consistent with genetic data from Drosophila [37].

A critical question left open concerns the substrate for MetAP-2 that is essential for noncanonical signaling. Previous studies have implicated members of the pertussis-sensitive Go family of heterotrimeric G proteins downstream of Fz receptors [24, 38]. In addition, Fz-mediated calcium release was shown to be pertussis sensitive [25]. Interestingly, Go, which is expressed in endothelial cells [39], is myristoylated and requires the action of a methionine aminopeptidase to expose the penultimate glycine residue for myristic acid coupling to its amino terminus. It is tempting to speculate that retention of the Go amino-terminal methionine upon TNP-470-mediated MetAP-2 inhibition could lead to a dominant-negative Go protein that subsequently blocks noncanonical Wnt signaling. Experiments to test this hypothesis are underway.

Another possible connection between MetAP-2 activity and noncanonical Wnt signaling may be via regulation of protein phosphatase PP2A. Previous studies have implicated a PP2A inhibitor as a substrate for methionine aminopeptidase activity [40]. Using a nonspecific MetAP inhibitor, bengamide E (3), Towbin and colleagues identified nine proteins whose mobility was altered upon MetAP inhibition. One of these proteins, a 42 kDa PP2A inhibitor, becomes more basic upon bengamide E incubation, consistent with the gain of a charged methionine amino terminus. Interestingly, wilderborst (wdb), a Drosophila mutant with aberrant planar polarization in wing epithelial cells was identified as a B regulatory subunit of PP2A [41]. This PP2A regulatory protein asymmetrically localizes to the distal side of a planar web of microtubules in the Drosophila wing epithelium. Genetic studies have shown wdb to be a conserved component of planar cell polarity in both Drosophila and zebrafish. It is interesting to note that, like Frizzled and Dishevelled, PP2A is another shared component in both canonical and noncanonical Wnt pathways. Studies have shown that PP2A is required for Wnt/β-catenin signaling in the developing embryo [42, 43]. While only speculative at this moment, it is possible that retention of the amino-terminal methionine of the PP2A inhibitor upon MetAP-2 inhibition could result in aberrant PP2A regulation, leading to loss of noncanonical Wnt signaling without affecting Wnt/β-catenin signaling. Epistasis studies with Drosophila MetAP-2 and wdb mutants may provide a connection between MetAP-2 and wdb. It is interesting to note that other genetic evidence, consistent with our conclusion that MetAP-2 plays an important role in noncanonical Wnt signaling, already exists. In support of our zebrafish and cell culture data, previous studies have shown that loss of MetAP-2 in Drosophila leads to an embryonic lethal phenotype; however, a weaker allele results in loss of ommatidial dorsal/ventral patterning in the eye, a characteristic phenotype of noncanonical Wnt signaling misregulation [44].

While other small-molecule agonists and antagonists of Wnt signaling have been reported [17, 18], we show that TNP-470 is the first, to our knowledge, small molecule capable of regulating noncanonical, but not canonical, Wnt signaling. Our finding that this potent antiangiogenic compound blocks noncanonical Wnt signaling...
clones were picked and analyzed by Western blot. A total of 1,3104 each of Rfz2-expressing mouse F9 cells and HEK293 cells coexpressing PE-specific marker cytokeratin Endo-A, immunofluorescent staining was performed according to the manufacturer’s recommended protocols. For staining of the primitive endoderm (PE)-specific marker cytokeratin Endo-A, immunofluorescent staining was carried out with TROMA-1 antibody (Developmental Studies Hybridoma Bank). Cells were rinsed with PBS, fixed with 3% paraformaldehyde at room temperature for 10 min, and permeabilized with 0.2% Triton for 5 min. After blocking in 10% donkey serum, cells were incubated with partially purified TROMA-1 antibody (1:10) in 1% BSA for 1 hr, followed by three washes (10 min each) with PBS. Samples were then incubated in the secondary antibody Rhodamine (TRICT)-conjugated anti-rat IgG (1:200) in 1% BSA at room temperature for 30 min, followed by three washes (10 min each) with PBS. Pictures were taken by a digital camera (Olympus C-3040ZOOM) linked to a fluorescent microscope (Olympus CK40).

siRNA Targeting Murine MetAP-2

Deoxyguanosine conjugates encoding siRNA against murine MetAP-2 were synthesized at the Keck Biotechnology Center (Yale School of Medicine). Sense oligonucleotide 5’-GGGTATAGACGACAGT-3’ and the antisense 5’-ACTGTCGCTATAGCCGACCTC-3’ were cloned into the BglII/HindIII sites of retrovirus pSUPER vector (OligoEngine). Retrovirus containing siRNA against murine MetAP-2 was produced with the PANTROPIC Retroviral Expression System (Clontech) and used to infect F9 cells before selection with 1 μg/ml puromycin.

[^3H]Thymidine Uptake Assay

Assays for thymidine uptake were carried out as previously described [45]. Each data point is the average of six values.

Luciferase Assay

F9 cells (20,000) expressing the pL-A/AR/Trz1 chimera were seeded into each well of 24-well plates 24 hr before transfection. Cells were transfected with the pTOPFlah plasmid by using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). After 16 hr, the cells were treated with isoproterenol (10 μM) for 6 hr, fumagillin (10 μM) for 2 hr, followed by fumagillin plus isoproterenol for another 6 hr or DMSO for 8 hr. Then, cells were harvested and analyzed with the Promega luciferase assay system, and luciferase activity was determined with the PerkinElmer Wallac 1420 multilabel counter. The fold increase was calculated as follows: Fold increase = 

Luc activity with iso after 6 hours – Luc activity before induction / Luc activity without iso after 6 hours – Luc activity before induction

CamKII Assay

CamKII activation in F9 cells was determined by using the Signa-TECT Calcium/Calmodulin-Dependent Protein Kinase (CamKII) Assay System (Promega Corporation). Briefly, 10^7 F9 cells carrying the pL-A/AR/Trz2 chimera with or without MetAP-2 knockdown by siRNA were treated with 100 μM isoproterenol for 10 min with or without pretreatment with 10 nM TNP-470 for 24 hr. Cells were lysed in 25 mM Tris-HCl (pH7.4), 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton, 10 mM β-mercaptoethanol, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. CamKII activity was determined by a [γ-32P]ATP in vitro kinase assay with bovine-tethered substrate that was performed according to the manufacturer’s protocols. Average values represent experiments done in triplicate.

RhoA Activity Assay

RhoA activity was determined by a GST-RBD pull-down assay with the EZ-Detect Rho Activation Kit (Pierce). Briefly, 10^6 MPE cells were preincubated with or without 10 nM TNP-470 for 16 hr, stimulated with Wnt8a conditioned medium for 1 hr, and washed with PBS. The assay was performed according to the manufacturer’s protocol.

Cloning and Whole-Mount In Situ Hybridization in Zebrafish

To clone zebrafish MetAP-2 homologs, human MetAP-2 was used as a query in a BLAST search of the zebrafish genomic database (http://wwwensembl.org/Danio rerio). As a result, two zebrafish MetAP-2 sequences were identified (on chromosome 4, and 25 [NCBI accession code: NM199640]). Gene-specific primers were designed and used to amplify MetAP-2 products from a 24 hr post fertilization cDNA library. PCR products were cloned into the TA Cloning Vector (InVitroGen) and confirmed by sequencing.
For whole-mount in situ hybridization, embryos were fixed in 4% paraformaldehyde x PBS. Digoxigenin-UTP-labeled antisense RNA probes (Roche) were synthesized by using SP6 RNA polymerase from Xhol-linearized MetAP-2 templates and T7 RNA polymerase from BamHI-linearized Wnt5 templates. After detection, embryos were mounted and photographed.

Morpholinos and Zebrafish Microinjection

Wild-type WIK and TLF zebrafish embryos were obtained by natural crosses. Embryos were microinjected with a pneumatic injector (WPI) at the one-cell stage. Morpholinos (Gene-Tools) for zebrafish MetAP-2 (Chromosome 25) (5'-CATGCTTTACCATCTTGACAG G-3'), the 5 bp mismatched (in lowercase letters) control missense MetAP-2 MO (5'-CATGTTTTACCATCTTGACAGG-3'), and Wnt5 (5'-GTCCTTGGTTCATTCTCACATCCAT-3') were injected at concentrations of 600 μM (MetAP-2 and missense MetAP-2) and 330 μM (Wnt5) in 1× Danieau solution. Synergy experiments were conducted at 300 μM and 150 μM for MetAP-2 and Wnt5, respectively. The single low-dose MO injections for the synergistic experiments were supplemented with control MO to bring up the total injected MO amount to a level equal to that of the combined Wnt5/MetAP-2 double MO injection experiments. In vitro-transcribed CaMKIItr (70–100 ng/μl) was cojected with MetAP-2 morpholinos. Injected embryos were reared at 28°C and assayed after 24 hr of development. Live embryos were mounted in methylcellulose, and images were captured at 5× magnification with a Zeiss axiocam.

Supplemental Data

Supplemental Data include zebrafish MetAP-2, MyoD, and Wnt5 expression analyses in wild-type and MetAP-2 MO-injected fish and are available at http://www.chembiol.com/cgi/content/full/13/9/1001/DC1/.

Acknowledgments

We thank Trudi Westfall for zebrafish crosses and embryo collection. We appreciate the many helpful discussions with Dr. Weimin Zhong and the critical reading of the manuscript provided by the members of the Crews lab. This work was supported by National Institutes of Health (NIH) grant CA030349 to C.M.C. and by American Cancer Society DDC-106889 and NIH grant CA112369 to D.C.S. The authors declare that they have no competing financial interests.

Received: May 16, 2006
Revised: June 26, 2006
Accepted: July 17, 2006
Published: September 22, 2006

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Accession Numbers

Zebrafish MetAP-2 Chromosome 4 sequence has been deposited in the National Center for Biotechnology Information with accession code DQ985554.