The β-catenin HMP-2 functions downstream of Src in parallel with the Wnt pathway in early embryogenesis of C. elegans

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The Wnt and Src pathways are widely used signal transduction pathways in development. β-catenin is utilized in both pathways, as a signal transducer and a component of the cadherin cell adhesion complex, respectively. A C. elegans β-catenin HMP-2 is involved in cell adhesion, but its signaling role has been unknown. Here, we report that in early embryogenesis HMP-2 acts as a signaling molecule in the Src signal. During early embryogenesis in C. elegans, the Wnt and Src pathways are redundantly involved in endoderm induction at the four-cell stage and spindle orientation in an ABaR blastomere. RNAi experiments demonstrated that HMP-2 functions in the Src pathway, but in parallel with the Wnt pathway in these processes. HMP-2 localized at the cell boundaries and nuclei, and its localization at cell boundaries was negatively regulated by SRC-1. In addition, HMP-2 was Tyr-phosphorylated in a SRC-1-dependent manner in vivo. Taken together, we propose that HMP-2 functions downstream of the Src signaling pathway and contribute to endoderm induction and ABaR spindle orientation, in parallel with the Wnt signaling pathway.

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I n t r o d u c t i o n

During development, some cell fates are induced by intercellular communications. In general, inducers secreted from neighboring cells interact with transmembrane receptors, which transduce the signals to nuclei so that the profiles of gene expression will be altered. Several evolutionarily conserved signal transduction pathways are known to be involved in induction, and in some cases multiple signaling pathways work cooperatively or redundantly. Among the signaling pathways that mediate intercellular communication, Wnt and Src pathways both utilize β-catenin (Nelson and Nusse, 2004). Wnt signaling regulates gene expression using β-catenin as a signal transducer (Logan and Nusse, 2004), and Src signaling regulates cell adhesion by Tyr phosphorylation of β-catenin in the cadherin complex on adherens junctions (Nelson and Nusse, 2004).

In the canonical Wnt pathway, Wnt pathway activation (e.g., through the receptor, Frizzled) inhibits β-catenin degradation (Huelsken and Behrens, 2002; Logan and Nusse, 2004). As a result, increased levels of cytoplasmic β-catenin become available for nuclear translocation. In the nucleus, β-catenin binds to T-cell factor/lymphoid enhancer factor (TCF/LEF) DNA-binding proteins and alters the transcription of target genes (Behrens et al., 1996).

Src is a family of cytoplasmic Tyr kinases, which interact with the cytoplasmic domain of transmembrane receptors and transmit signals by phosphorylating other proteins. During cell–cell adhesion, the intracellular domain of cadherin binds directly to β-catenin. Src phosphorylates a particular Tyr residue (Y654) of β-catenin (Lilien and Balsamo, 2005), consequently disrupting β-catenin binding to cadherin and leading to the loss of adherens junctions (Daugherty and Gottardi, 2007; Lilien and Balsamo, 2005). The fate of cadherin-bound β-catenin after dissociation from the adherens junction is not well understood, but it is generally agreed that it is either degraded or recycled (Bryant and Stow, 2004).

During early embryogenesis in Caenorhabditis elegans, the Wnt and Src pathways are redundantly involved in endoderm induction and mitotic spindle orientation (Fig. 1). Endoderm induction in C. elegans occurs at the four-cell stage. The posterior-most cell, P2, induces its anterior sister cell, EMS, to undergo asymmetric division and produce an anterior descendant, MS, which produces primarily mesoderm, and a posterior descendant, called E, that gives rise to the entire endoderm (Goldstein, 1995) (Figs. 1A and Ba). During endoderm induction, Wnt signaling brings β-catenin WRM-1 into the E nucleus and exclude POP-1 (TCF/LEF) from the nucleus, resulting in enrichment of POP-1 in the MS nucleus compared to the E nucleus (Fig. 1Ca) (Rocheleau et al., 1999). Unlike the canonical Wnt pathway in which TCF/LEF induces the transcription of target genes, POP-1 represses the transcription of E-specific genes in MS (Calvo et al., 2001; Maduro et al., 2002). Single mutants of Wnt signaling components cause the cell fate transformation of E to
its sister blastomere MS resulting in the absence of endoderm with incomplete penetrance (Bei et al., 2002; Lin et al., 1995; Rocheleau et al., 1997, 1999; Thorpe et al., 1997), while single mutations of Src pathway components do not cause such defects (Bei et al., 2002). When both a Wnt signaling component and a Src signaling component are mutated the penetrance of the defect in endoderm induction is enhanced (Bei et al., 2002).

Only two Src signaling components, the Tyr kinase receptor MES-1 and the Src homolog SRC-1, have been identified in this process. MES-1 accumulates at the P2–EMS boundary, where endoderm induction occurs (Berkowitz and Strome, 2000). Strong tyrosine phosphorylation (pTyr) is detected at the P2–EMS boundary, and SRC-1 and MES-1 are required for the accumulation of pTyr (Bei et al., 2002). Recently, auto-phosphorylated SRC-1 was shown to localize at the P2–EMS boundary, but the target of SRC-1 is unknown (Liu et al., 2010). Therefore, Src signaling positively regulates endoderm induction, probably through Tyr phosphorylation of an unknown target(s) at the P2–EMS boundary in parallel with activation of Wnt signaling. However, the downstream components of the Src pathway, including proteins phosphorylated at the P2–EMS boundary remain to be identified.

The functional redundancy of the Wnt and Src pathways is also observed in the regulation of spindle orientation of the ABar blastomere (Figs. 1Bb and Cb) (Bei et al., 2002; Walston et al., 2004). Wnt and Src signals from a C blastomere to an ABar blastomere alter the spindle orientation in ABar (Fig. 1Bb) (Bei et al., 2002; Walston et al., 2004). Many components are shared between spindle orientation and endoderm induction (Fig. 1Cb).

C. elegans expresses at least four β-catenins, namely BAR-1, WRM-1, SYS-1, and HMP-2, with different functions (Kidd et al., 2005; Korswagen et al., 2000; Liu et al., 2008; Natarajan et al., 2001). BAR-1, WRM-1, and SYS-1 play roles in Wnt signaling; BAR-1 is involved in multiple cell fate determination events during postembryonic development (Malloof et al., 1999), whereas WRM-1 and SYS-1 act in postembryonic and embryonic cell fate determination events including endoderm induction (Huang et al., 2007; Rocheleau et al., 1999) in parallel with the Src pathway. On the other hand, HMP-2 has been thought to be specialized for cell adhesion. HMP-2 interacts with
membrane-bound HMR-1 (cadherin ortholog) and HMP-1 (α-catenin ortholog) and is involved in adhesion as in other animals (Costa et al., 1998; Korswagen et al., 2000; Natarajan et al., 2001). Because HMP-2 has not been detected in the nuclei of endoderm precursors and disruption of HMP-2 does not cause endoderm induction defect (Putzke and Rothman, 2010), HMP-2 has not been considered to be involved in Wnt signaling.

Here, we report that HMP-2 is a signaling molecule in the Src pathway during endoderm induction and in mitotic spindle orientation in ABar cells in early C. elegans embryos. We found that Src signaling induces Tyr phosphorylation of HMP-2, and phosphorylated HMP-2 accumulates in nuclei of early embryonic cells including E and MS. We propose that HMP-2 functions downstream of the Src signaling and contributes to endoderm induction and spindle orientation in concert with the Wnt signaling.

Material and methods

Strains and culture

Strains of C. elegans were derived from the wild-type Bristol strain N2 and cultured as described (Brenner, 1974). Strains used were wild-type strain N2, med-1::gfp::pop-1 (JR1704; Maduro et al., 2002), tbl-35::gfp (MS52; Broitman-Maduro et al., 2006), end-3::END-3[P202L]; gfp (JR2274; Maduro et al., 2005), src-1(c293) (WM65; Bei et al., 2002), mom-2(or309) (HS1754; Sugioaka and Sawa, 2010). N2 and src-1(c293) were maintained at 20 °C. The other strains were maintained at 24 °C.

RNA interference

RNAi was performed as described (Fire et al., 1998). PCR-amplified fragments from cDNA clones (yk55h11 for dsh-2, yk46b11 for mig-5, yk16a11 for src-1, and yk434b12 for hmp-2) and the C. elegans cDNA library (Hayashizaki et al., 1998) (for hmr-1 and hmp-1) were used as templates to prepare double-stranded RNA. Although wrm-1, another β-catenin, is known to be required for endoderm induction, wrm-1 and hmp-2 share only 43% nucleotide identity thus hmp-2(RNAi) would not interfere with wrm-1 gene expression. Double-stranded RNA was microinjected into the gonad or intestine of wild type, med-1::gfp::pop-1, tbl-35::gfp, and end-3::END-3[P202L]; gfp young adult hermaphrodites. Injected worms (F1 generation) and their progeny (F1 generation) were grown at 24 °C and collected 24 h after the injection. F1 embryos were examined for phenotypes.

Immunofluorescence and DAPI staining

Embryos were processed for staining as described (Miller and Shakes, 1995), with modifications. Briefly, embryos were permeabilized by the freeze and crack method. Embryos were fixed in methanol for 5 min at −30 °C and rehydrated with two washes in PBS followed by a wash in PBST (phosphate-buffered saline containing 0.5% (v/v) Tween-20). For anti-HMP-2 staining, embryos were treated with blocking solution (1% skim milk (Mornigana, Japan), 0.5% fetal bovine serum in PBST) for 30 min at room temperature. For anti-phospho-Tyr pY99 staining, blocking was omitted. Embryos were incubated overnight at 4 °C with the primary antibody in blocking solution (for pY99 staining, 5% fetal bovine serum in PBST), then with the secondary antibody for 1–2 h at room temperature. The samples were mounted for fluorescence microscopy with Vectashield containing 1.5 μg/ml DAPI (Vector Laboratories, USA).

Antibodies used were: anti-HMP-2 sc-15520 (Grana et al., 2010; Zaidel-Bar et al., 2010) and pY99 (anti-phospho Try) (Bei et al., 2002) (both from Santa Cruz Biotechnology, USA), and FITC-conjugated donkey anti-goat (115-095-003, Jackson ImmunoResearch, USA) and FITC-conjugated goat anti-mouse (705-095-147, Jackson ImmunoResearch, USA).

Microscopy

Live organisms and immunostained samples were examined using a Zeiss (Germany) Axioskop microscope equipped with Nomarski differential interference contrast and epifluorescence optics, a Zeiss LSM 510 confocal microscope, or a Zeiss Axioplan2 microscope equipped with Nomarski differential interference contrast and epifluorescence optics. All photographs for each series of experiments were taken under the same conditions. Epifluorescence microscopy images were acquired with an AxioCam CCD camera (Zeiss, Germany) and processed using AQUA Lite 1.2 (Hamamatsu, Japan) and Adobe Photoshop software. Confocal microscopy images were converted to tiff images using ImageJ software (Wayne Rasband, USA) and processed with Adobe Photoshop software.

Image analysis

Fluorescence intensity was quantified using ImageJ software. The fluorescence intensity was calculated by subtracting averaged pixel value in the background from averaged pixel value in the quantified area. For nuclear GFP signals in E and MS (med-1::GFP::POP-1), in Ea and Ep (end-3::END-3::GFP), and nuclear signals of anti-HMP-2 and pY99 staining, the fluorescence intensity of an area of 14.00 μm² was measured. Because the ectopic tbl-35::GFP signals were not concentrated in the nuclei of Ea and Ep cells in dsh-2(RNAi); mig-5(RNAi) and dsh-2(RNAi); mig-5(RNAi); hmp-2(RNAi) embryos, the fluorescence intensity of a cytoplasmic area of 14.00 μm² was measured. For quantification of fluorescent intensity of cell–cell boundaries, pixel values of a small rectangular area (7.47 μm²) on the boundary were measured, and the values of the background were subtracted.

Statistical analysis was performed with Microsoft Excel software and MEPHAS (http://www.gen-info.osaka-u.ac.jp/testdocs/tomo.com/). Wilcoxon signed-rank test was used to compare paired data, and Mann–Whitney U test was used to compare non-paired data.

Western blotting

SDS-soluble total nematode extracts were run on a NuPAGE (Invitrogen, USA) 7% Tris–acetate gel and blotted onto a Hybond P PVDF membrane (GE Healthcare, USA). The membrane was blocked with CANGETSIGNAL blocking solution (Nichiyu, Japan) overnight at 4 °C and probed with pY99 in CANGETSIGNAL solution 1 (TOYOBO, Japan) at room temperature. The membrane was washed three times with TBST (0.9% NaCl, 20 mM Tris–HCl, pH 7.4, 0.05% (v/v) Tween 20) at room temperature and probed with horseradish peroxidase-conjugated anti-mouse (Santa Cruz Biotechnology, USA) 1:2000 diluted with CANGETSIGNAL solution 2 (TOYOBO, Japan), followed by three washes with TBST at room temperature. The signal was then detected with ECLplus (GE Healthcare, USA) and Image analyzer LAS1000 (Fuji Film, Japan). After detection, the membrane was washed with TBST at room temperature and treated with Reblot plus strong solution (Chemicon, Japan) for 15 min at room temperature, then reprobed with anti-HMP-2 (Santa Cruz Biotechnology, USA) with the same procedure above.

To detect the mobility shift by phosphorylation, Western blotting following electrophoresis on an acrylamide gel with 50 μM Phos-tag acrylamide was carried out as described (Kinoshita et al., 2006). Phos-tag is a phosphate-binding molecule and its incorporation in the gel enhances the mobility shift of the phosphorylated protein in the gel (Kinoshita et al., 2006). SDS-soluble total nematode extracts were run on a 7.5% acrylamide resolution gel and blotted to Hybond P PVDF membrane at 6 V for 16 h at 4 °C. The membrane was blocked with...
components with the endoderm induction pathway, we examined

**HMP-2 is involved in endoderm induction**

suggest that, in the regulation of ABar spindle orientation,

hmp-2

involved in different pathways. On the other hand, double RNAi of
dsh-2(RNAi) (Fig. 2B), indicating that

- **hmp-2**
- **dsh-2(RNAi)**

may be involved in the spindle orientation defect in RNAi-treated embryos for the Wnt and Src pathway genes.

CANGETSIGNAL blocking solution for 30 min at room temperature and then probed with anti-HMP-2 as described above.

**Results**

**Screening of genes involved in the Wnt and Src pathways identified the β-catenin hmp-2**

Disruption of a single component of the Wnt or Src pathway causes a spindle orientation defect in ABar blastomeres with incomplete penetrance, whereas inhibition of both pathways significantly enhance the penetrance of the phenotype (Walston et al., 2004) (Figs. 2A and B). To identify new components in the Src pathway, we first screened for genes required for ABar spindle orientation.

First, genes expressed in early embryos were selected from our in situ hybridization database (The Nematode Expression Pattern Database, http://nematode.lab.nig.ac.jp/). The selected 1243 genes were next examined by RNAi for embryonic lethality. 178 genes that showed over 50% embryonic lethality were selected, and their early embryogenesis was recorded by 4D Nomarski microscopy. The 4D movies were visually analyzed for the defect in the spindle orientation of ABar cell. The hmp-2 gene was included in the 21 genes that showed ABar spindle orientation defect with moderate penetrance (Figs. 2Ac and B), which is similar to the RNAi of dsh-2 (Disheveled) and src-1 (Src) genes. Double RNAi of hmp-2 and dsh-2 increased the penetrance of the spindle orientation defect, similar to src-1(RNAi); dsh-2(RNAi) (Fig. 2B), indicating that hmp-2 and dsh-2 may be involved in different pathways. On the other hand, double RNAi of hmp-2 and src-1 did not enhance the defect (Fig. 2B). These results suggest that, in the regulation of ABar spindle orientation, hmp-2 may be involved in the Src pathway, but not in the Wnt pathway.

**HMP-2 is involved in endoderm induction**

Because the ABar spindle orientation pathway shares many components with the endoderm induction pathway, we examined whether hmp-2 is also involved in endoderm induction. The endoderm induction defect can be detected by the lack of gut granules, which emit bright fluorescence under UV light, at the terminal stage (“gutless” phenotype) (Lauger et al., 1980). Whereas the knock-down of Wnt pathway causes the gutless phenotype with moderate penetrance, the knock down of the Src pathway does not cause a gutless phenotype. However, the knock down of Src pathway along with Wnt pathway greatly enhances the penetrance of a gutless phenotype (Fig. 3A; Bei et al., 2002). Among embryos with disrupted Wnt pathway components, a double disruptant of two redundant Disheveled genes, dsh-2 and mig-5, are highly sensitive to Src pathway disruption (Bei et al., 2002; Thorpe et al., 1997) (Fig. 3A), and dsh-1, the other Disheveled, of which expression is low in early embryos (Baugh et al., 2003) does not enhance the endoderm induction defect of dsh-2(RNAi);mig-5(RNAi) (Walston et al., 2004).

The single knockdown of hmp-2 did not cause the gutless phenotype, similar to RNAi of dsh-2;mig-5, mom-5 (Frizzled), or src-1 (Fig. 3A). Next we tested the effect of hmp-2(RNAi) on the gutless phenotype of RNAi embryos of the Wnt or Src pathway genes. RNAi of hmp-2 enhanced the gutless phenotype of dsh-2(RNAi);mig-5(RNAi) embryos from a penetrance of 3% to 32% (Fig. 3A). Moreover, hmp-2 (RNAi) moderately enhanced the gutless phenotype of mom-5(RNAi) embryos (Fig. 3A). Thus, HMP-2 appears to function redundantly with the Wnt pathway in endoderm induction. In contrast, src-1(RNAi); hmp-2(RNAi) induced the gutless phenotype with very low penetrance, suggesting that HMP-2 is likely to contribute to endoderm induction via the Src pathway, although HMP-2 may have a minor role in endoderm induction outside the Src pathway (Fig. 3A). Collectively, as in an ABar spindle orientation process, HMP-2 apparently functions in the Src pathway independently of the Wnt pathway in endoderm induction.

**E descendants are partially transformed to the MS fate in dsh-2(RNAi); mig-5(RNAi);hmp-2(RNAi) embryos**

It has been shown that, when both Wnt and Src pathways are blocked, E descendants lose their characteristics and acquired MS-like characteristics (Broitman-Maduro et al., 2006; Lin et al., 1995; 1998; Maduro et al., 2005; Shetty et al., 2005). We asked whether the gutless phenotype observed in dsh-2(RNAi);mig-5(RNAi);hmp-2(RNAi) embryos were caused by the same E-to-MS transformation, by observing E descendants are partially transformed to the MS fate in dsh-2(RNAi); mig-5(RNAi);hmp-2(RNAi) embryos.

We first observed the expression of end-3 in E descendants using the end-3::END-3[P202L]::GFP strain (Maduro et al., 2005), end-3 and its closely related homolog end-1 are the earliest genes expressed in the E lineage (Figs. 3Ba and b) and are key transcription factors in determining endoderm fate. Ectopic expression of end-3 or end-1 results in ectopic endoderm differentiation, and disruption of both end-3 and end-1 abrogates endoderm formation (Maduro et al., 2005). We measured the fluorescence intensity of END-3[P202L]::GFP in the nucleus of Ea or Ep. The GFP signal in Ea or Ep was significantly reduced in dsh-2(RNAi);mig-5(RNAi);hmp-2(RNAi) embryos compared to untreated embryos and dsh-2(RNAi);mig-5(RNAi) embryos (Figs. 3Bc-e).

We next observed the expression of the earliest MS marker gene, txb-35, using the txb-35::GFP strain (Broitman-Maduro et al., 2006) to see if E descendants acquired the characteristics of MS descendants. Depletion of txb-35 causes loss of MS-derived tissues, and its overexpression directs all cells to the MS fate (Broitman-Maduro et al., 2006). Whereas the txb-35::GFP signal was barely detected in E lineage cells of the dsh-2(RNAi);mig-5(RNAi) embryos, dsh-2(RNAi); mig-5(RNAi);hmp-2(RNAi) embryos ectopically expressed txb-35::GFP in the cytoplasm and nuclei of Ea and Ep (Figs. 3Cc-e).

Finally, we examined the asymmetry of POP-1 protein in nuclei of E and MS cells. The Wnt and Src pathways are known to redundantly regulate endoderm induction by making asymmetric distribution of
PO-1 in nuclei of E and MS cells (Bei et al., 2002; Rocheleau et al., 1997; Thorpe et al., 1997), which are established by excluding PO-1 from the E nucleus (Lo et al., 2004). We quantified nuclear PO-1 in MS and E by measuring the nuclear fluorescence intensity of GFP::PO-1 using the med-1::gfp::pop-1 strain, which expresses GFP::PO-1 in EMS descendants (Maduro et al., 2002) (Figs. 3Da–e). The fluorescence intensity of nuclear GFP::PO-1 in E, as well as the E/MS ratio, was significantly higher in dsh-2(RNAi);mig-5(RNAi);hmp-2
SRC-1 reduces HMP-2 on P2/EMS boundary

Because HMP-2 appears to be involved in endoderm induction, we analyzed the localization of HMP-2 in the four-cell embryos when the induction occurs. HMP-2 was shown to localize to the cell–cell boundary in early embryos (Costa et al., 1998). By immunofluorescence, we found that the anti-HMP-2 signal was detected on the P2–EMS boundary at a lower level than on the ABA–EMS boundary (Fig. 4Aa, Ba and b). In hmp-2(RNAi) embryos, the cortical signals both at the ABA–EMS and P2–EMS boundaries were greatly reduced compared to untreated embryos (Figs. 4Ab and Ba), demonstrating that the signal indeed corresponds to the endogenous HMP-2 protein. Because HMP-2 seems to be involved in the Src pathway, we examined whether the asymmetric cortical localization of HMP-2 would be affected by inhibiting the Src pathway. We found that fluorescence intensity of anti-HMP-2 signal at the P2–EMS boundary was significantly increased in src-1(RNAi) embryos (Figs. 4Ac and Ba), resulting in the loss of asymmetry between the ABA–EMS boundary and the P2–EMS boundary (Fig. 4Bb). Therefore SRC-1 appears to be involved in reducing HMP-2 on the P2–EMS boundary.

HMP-2 may be Tyr phosphorylated by SRC-1 on P2/EMS boundary in a dependent manner

SRC-1 is a major Tyr kinase at four-cell stage and is required for Tyr phosphorylation at cell–cell boundaries in four-cell embryos (Beili et al., 2002). The pY99 antibody that recognizes phosphorylated tyrosine stains all cell–cell boundaries in four-cell embryos, with strong signal at the P2–EMS boundary where endoderm induction occurs (Beili et al., 2002) (Fig. 4Ad). This phospho-Tyr signal of unknown protein(s) requires SRC-1 (Beili et al., 2002) (Figs. 4Af and Bc) and is considered to reflect the strong activity of SRC-1 at the P2–EMS boundary (Beili et al., 2002).

Because HMP-2 is reduced by SRC-1 from P2/EMS boundary, we speculated that HMP-2 might be Tyr phosphorylated by SRC-1 on P2–EMS boundary. In hmp-2(RNAi) embryos, the pY99 signals at both ABA–EMS and P2–EMS boundaries were reduced compared to untreated embryos and the reduction on P2–EMS boundary was prominent (Figs. 4Ac, Bc and e), raising the possibility that HMP-2 is a major protein phosphorylated at Tyr residues on P2/EMS boundary in a SRC-1-dependent manner.

SRC-1 promotes nuclear localization of HMP-2 in E, the endoderm progenitor cell

During the analysis of HMP-2 localization in early embryos, we noticed that anti-HMP-2 stained nearly all nuclei in two- to twelve-cell stage embryos (Fig. 5Aa). The nuclear anti-HMP-2 signals as well as cortical signal were greatly reduced in hmp-2(RNAi) embryos (Figs. 5Ab and Ba), confirming that this signal reflects the distribution of endogenous HMP-2 proteins. Because localization of HMP-2 on cell–cell boundaries is regulated by SRC-1, we examined the effect of src-1(RNAi) on nuclear localization of HMP-2. Nuclear HMP-2 was diminished in early src-1(RNAi) embryos (Figs. 5Ac and Ba) indicating that SRC-1 promotes nuclear localization of HMP-2. Therefore, HMP-2 seems to move between cell boundary and nucleus, and this dynamic change may be regulated by SRC-1.

We also found that the pY99 antibody stained nearly all the nuclei of two- to twelve-cell stage embryos in addition to the reported cortical staining (Figs. 5Ae and Bb) (Beili et al., 2002). All the cortical and nuclear signals including signal in E nucleus were greatly diminished in src-1(RNAi) embryos compared to that in untreated embryos (Figs. 5Ag and Bb), indicating that these cortical and nuclear signals reflect SRC-1-dependent Tyr phosphorylation. The nuclear pY99 signal was also greatly reduced in hmp-2(RNAi) embryos (Figs. 5Af and Bb). These results imply that HMP-2 is a major protein phosphorylated in a SRC-1-dependent manner on the P2/EMS boundary and E nucleus, and raise an intriguing possibility that SRC-1 might affect the dynamic change of HMP-2 localization through Tyr phosphorylation.

Tyr residue in HMP-2 is phosphorylated in a SRC-1-dependent manner

In other organisms, Src regulates localization and activity of β-catenin through Tyr phosphorylation (Daugherty and Gottardi, 2007; Lilien and Balsamo, 2005) and our analysis revealed that the localization of HMP-2 is regulated by SRC-1, possibly by Tyr phosphorylation. To investigate SRC-1-dependent phosphorylation of HMP-2, whole adult worm extracts of untreated worms and src-1 mutants were analyzed by western blotting with anti-HMP-2 and anti-phospho-Tyr pY99 (Fig. 6A). The band detected by anti-HMP-2 and one of the bands detected by pY99 overlapped. The intensity of the pY99 band that overlapped with the HMP-2 band was greatly reduced in src-1 mutant extracts, even though the intensity of the HMP-2 band was not reduced compared to the untreated extract (Fig. 6A). This indicates a protein with the same molecular weight as that of HMP-2 is Tyr-phosphorylated in a SRC-1-dependent manner.

We also performed western blotting of wild-type and src-1 mutant extracts with anti-HMP-2 using a Phos-tag acrylamide gel (Fig. 6B) to further confirm the SRC-1-dependent phosphorylation of HMP-2. Phos-tag binds to phospho groups of phosphorylated proteins and retards their mobility during electrophoresis (Kinoshiba et al., 2006), thereby the band shift of phosphorylated protein is magnified. An upwardly shifted HMP-2 band was detected in the Phos-tag gel in wild-type extracts, whereas this phosphorylated band was very weak in src-1 mutant extracts. Thus, HMP-2 appears to be phosphorylated in a SRC-1-dependent manner in vivo, although whether this is the direct phosphorylation is unclear from these experiments.

Depletion of cadherin restores nuclear HMP-2 and facilitates endoderm formation

Like β-catenins in other organisms, HMP-2 is thought to be anchored to the cortex by binding to HMR-1 (cadherin) (Costa et al.,...
Phosphorylation of mammalian β-catenin by Src disrupts the interaction between β-catenin and cadherin and causes release of β-catenin from cortex. Thus, by analogy, Tyr phosphorylation of HMP-2 by SRC-1 may cause nuclear accumulation of HMP-2 by release of HMP-2 from HMR-1. If it is the case, depletion of HMR-1 should cause nuclear localization of HMP-2 in the absence of SRC-1. Indeed, immunostaining of src-1(RNAi);hmr-1(RNAi) embryos with anti-HMP-2 revealed that HMR-1 depletion significantly increased the amount of nuclear HMP-2 in E, compared to src-1(RNAi) embryos (Figs. 5Ad and Ba). Nuclear

**Fig. 4.** Localization of HMP-2 and phospho-Tyr at the cell–cell boundary of four-cell embryos. (A) (a–c) Confocal microscopy images of four-cell stage embryos stained with anti-HMP-2. (a) Untreated, (b) hmp-2(RNAi) and (c) src-1(RNAi). (d–f) Confocal microscopy images of four-cell embryos stained with anti-phospho Tyr (pY99). (d) Untreated, (e) hmp-2(RNAi), and (f) src-1(RNAi). Red arrowheads indicate the ABa–EMS boundary and blue arrowheads indicate the P2–EMS boundary. Bar: 10 μm. (B) (a, c) Scatter plot showing fluorescence intensity (arbitrary unit) of (a) anti-HMP-2 and (c) pY99 at the ABa–EMS boundary (red) and the P2–EMS boundary (blue) in four-cell-stage untreated, hmp-2(RNAi), and src-1(RNAi) embryos. (b, d) Scatter plot showing the A/P ratio of fluorescence intensity of (b) anti-HMP-2 and (d) pY99 in four-cell embryos. Horizontal bars in scatter plots indicate the average values. *p < 0.05; **p < 0.01; Mann–Whitney U test. ††p < 0.01; Wilcoxon signed-rank test.
phospho-Tyr remained at low levels in the src-1(RNAi);hmr-1(RNAi) embryos (Figs. 5Ah and Bb), indicating that the nuclear HMP-2 was not phosphorylated in the absence of SRC-1 and HMR-1.

If release of HMP-2 from HMR-1 leads to nuclear accumulation of HMP-2, and nuclear HMP-2 contributes to endoderm induction, increasing nuclear HMP-2 by reducing HMR-1 may facilitate the endoderm in the absence of Wnt signaling. Indeed, hmr-1(RNAi) partially suppressed the gutless phenotype of mom-2(or309) mutant in which the C-terminal half of MOM-2 (Wnt) is deleted (78% in mom-2(or309) (n=145); 53% in mom-2(or309);hmr-1(RNAi) (n=116)).

Taken together, we propose a model that SRC-1 promotes release of HMP-2 from HMR-1 by Tyr-phosphorylation of HMP-2 and thereby cause nuclear localization of HMP-2 and contributes to endoderm induction (Fig. 7).

Discussion

In this paper, we identified a C. elegans β-catenin HMP-2 as a component in the Src pathway that is involved in ABar spindle orientation and endoderm induction. In contrast to other β-catenin proteins in C. elegans function, we found that HMP-2 works independently of the Wnt pathway in these processes. Based on our data, we propose the following model for the endoderm induction (Fig. 7); SRC-1-dependent Tyr phosphorylation of HMP-2 causes release of HMP-2 from P2–EMS boundary. After division of an EMS cell, phosphorylated HMP-2 became enriched in the E nucleus. We speculate that HMP-2 that is accumulated in the E nucleus contributes to reduction of nuclear POP-1, which eventually induces endoderm fate.

Crosstalk between the Wnt and Src pathways via β-catenin

Because the Wnt and Src pathways share β-catenin as a major component in many organisms, an attractive hypothesis is that β-catenin released from cadherin in response to Src stimulation activates the Wnt pathway (Nelson and Nusse, 2004). It was shown that when β-catenin is artificially released from cadherin complex, β-catenin is able to activate the Wnt pathway. In C. elegans, when HMP-2 remains at low levels in the src-1(RNAi);hmr-1(RNAi) embryos (Figs. 5Ah and Bb), indicating that the nuclear HMP-2 was not phosphorylated in the absence of SRC-1 and HMR-1.

If release of HMP-2 from HMR-1 leads to nuclear accumulation of HMP-2, and nuclear HMP-2 contributes to endoderm induction, increasing nuclear HMP-2 by reducing HMR-1 may facilitate the endoderm in the absence of Wnt signaling. Indeed, hmr-1(RNAi) partially suppressed the gutless phenotype of mom-2(or309) mutant in which the C-terminal half of MOM-2 (Wnt) is deleted (78% in mom-2(or309) (n=145); 53% in mom-2(or309);hmr-1(RNAi) (n=116)). Taken together, we propose a model that SRC-1 promotes release of HMP-2 from HMR-1 by Tyr-phosphorylation of HMP-2 and thereby cause nuclear localization of HMP-2 and contributes to endoderm induction (Fig. 7).

Discussion

In this paper, we identified a C. elegans β-catenin HMP-2 as a component in the Src pathway that is involved in ABar spindle orientation and endoderm induction. In contrast to other β-catenin proteins in C. elegans function, we found that HMP-2 works independently of the Wnt pathway in these processes. Based on our data, we propose the following model for the endoderm induction (Fig. 7); SRC-1-dependent Tyr phosphorylation of HMP-2 causes release of HMP-2 from P2–EMS boundary. After division of an EMS cell, phosphorylated HMP-2 became enriched in the E nucleus. We speculate that HMP-2 that is accumulated in the E nucleus contributes to reduction of nuclear POP-1, which eventually induces endoderm fate.

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is released from cadherin complex by knockdown of FRK-1, which is necessary to anchor HMP-2 to cadherin, HMP-2 was shown to substitute the function of another $\beta$-catenin WRM-1 in endoderm induction (Putzke and Rothman, 2010). A study of a mammalian cell line revealed that when $\beta$-catenin is artificially released from cadherin by drug treatment, cadherin-bound $\beta$-catenin is recruited to the nucleus to induce the Wnt signal response (Kam and Quaranta, 2009). However, crosstalk between the Wnt and Src pathways via $\beta$-catenin in physiological process has not been shown. Our results in C. elegans embryos provide an example of crosstalk between the Wnt and Src pathways in which $\beta$-catenin in the cadherin complex functions downstream of the Src signal and merge into the Wnt pathway in normal development.

In other organisms, Tyr phosphorylation of $\beta$-catenin increases its ability to act as a transcriptional co-activator (Daugherty and Gottardi, 2007; Lilien and Balsamo, 2005). Our results raise another potential role

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**Fig. 6.** SRC-1-dependent Tyr phosphorylation of HMP-2. (A) Western blotting of wild-type (WT) and src-1(cj293) mutant worm extracts with anti-HMP-2 (green) and phospho-Tyr (pY99) (red). Asterisks indicate the position of the HMP-2 band. (B) Western blotting of wild-type and src-1(cj293) mutant worm extracts with anti-HMP-2 using acrylamide gel containing Phos-tag that binds to phosphate to enhance the mobility shift. Two lanes from the same membrane are combined and shown. The arrowhead indicates the HMP-2 band, and the arrow indicates SRC-1-dependent shifting of the HMP-2 band. Asterisk in src-1 lane indicates the position of a shifted band in the wild-type. Although the band is distorted due to the effect of Phos-tag in the gel, an upshifted band is detected only in the wild-type lane.

**Fig. 7.** Schematic representation of the roles of SRC-1 and HMP-2 in endoderm induction.
of Tyr phosphorylation for the transcriptional regulation by release of β-catenin from the cadherin complex into cytoplasmic β-catenin pool (then to nuclei), and this phosphorylation-dependent relocation of β-catenin, rather than phosphorylation-dependent increase of transcriptional activity, may be crucial for affecting downstream transcription. Consistent with this hypothesis, our preliminary data indicates that depletion of the cadherin, HMR-1, was sufficient to bypass Wnt and Src signaling and that HMP-2 induced endoderm fate without being Tyr phosphorylated (E.S. unpublished data). Further analysis of HMP-2 phosphorylation including identification of phosphorylation sites will clarify its role in the downstream events.

Another SRC-1 target that redundantly acts with HMP-2

We found that the phenotypes of dsh-2(RNAi); mig-5(RNAi); hmp-2 (RNAi) embryos are weaker than those of dsh-2(RNAi); mig-5(RNAi); src-1(RNAi) embryos; 30% of the former showed the gutless phenotype, compared to 80% in the latter (Fig. 3A), and only partial effects on POP-1 localization, and downstream gene expression were observed in dsh-2(RNAi); mig-5(RNAi); hmp-2(RNAi) embryos (Figs. 3B, C and D). Thus, HMP-2 depletion did not fully abolish the Src signaling that induces endoderm formation. Although src-1(RNAi) abolished nearly all the staining with the anti-β-phospho Tyr antibody (pY99) (Fig. 5A), hmp-2(RNAi) did not eliminate all the pY99 staining. The signals at the cell–cell boundaries were retained in hmp-2(RNAi) embryos, especially at the eight-cell stage (Fig. 5A). Furthermore, western blotting showed that many proteins in addition to HMP-2, were phosphorylated in a SRC-1-dependent manner (Fig. 6A). Therefore, SRC-1 is likely to phosphorylate proteins other than HMP-2 that may redundantly regulate endoderm induction. One candidate substrate may be another cadherin complex component, p120 catenin homolog JAC-1 (Pettitt et al., 2003). In vertebrates, the cadherin binding activity of p120 catenin, is regulated by Src, and phosphorylation of p120 catenin by Src translocates the protein to the nucleus where it regulates the DNA binding and transcriptional activity of Kaiso (Daniel, 2007). Although JAC-1 mutants do not show endoderm induction defects (Pettitt et al., 2003), the effect of JAC-1 disruption on the function of Wnt pathway components is unknown; genetic interaction studies involving JAC-1 and Wnt pathway components may help to clarify this point.

SRC-1 may directly phosphorylate HMP-2

In mammals, Src directly phosphorylates β-catenin (Lilien and Balsamo, 2005). Src-mediated phosphorylation of the conserved Tyr residue (Y654) in the armadillo repeat causes release of β-catenin from cadherin (Daugherty and Gottardi, 2007; Lilien and Balsamo, 2005). We have shown SRC-1-dependent Tyr phosphorylation of HMP-2 in vivo, which likely causes release of HMP-2 from cadherin HMR-1. Furthermore, HMP-2 contains a conserved Tyr corresponding to Y654. Therefore, it is plausible that SRC-1 directly phosphorylates this conserved Tyr to regulate the interaction between HMP-2 and HMR-1 in C. elegans.

How does HMP-2 induce endoderm formation?

Although HMP-2 has been reported not to bind to POP-1 using the yeast two-hybrid system and immunoprecipitation (Korswagen et al., 2000), several lines of evidence suggest HMP-2 can interact with POP-1 in vivo. First, ectopic overexpression of HMP-2 rescues the mutant phenotype of bar-1 encoding another β-catenin that binds POP-1 and promotes its nuclear translocation and activation of transcriptional activity (Natarajan et al., 2004). Second, HMP-2 can substitute the endoderm inducing activity of yet another β-catenin WRM-1, which binds POP-1 to exclude POP-1 from the nucleus, in the absence of FRK-1 (Putzke and Rothman, 2010). Therefore, it is reasonable to assume that HMP-2 directly binds to POP-1, as seen for BAR-1 and WRM-1, to affect the localization and/or activity of POP-1. Further characterization of interaction between HMP-2 and POP-1 will reveal the functional redundancy and diversity of β-catenin families in the crosstalk between Wnt and Src pathways.

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