Introduction

The Wnt family of secreted glycoproteins plays a critical role in developmental processes including axis patterning, cellular proliferation, planar cell polarity, cell migration, cell fate specification, and neuronal development. In adults, Wnt signaling is involved in tissue homeostasis, regeneration, and stem/progenitor cell function. Moreover, elevated or attenuated Wnt signaling is found in a diversity of diseased tissues (Logan and Nusse, 2004; Moon et al., 2004).

Wnt signaling can be divided into CTNNB1-dependent and CTNNB1-independent signaling. The best-characterized family of receptors for Wnts is the Frizzled class of seven-pass transmembrane atypical G protein–coupled receptors. In addition, CTNNB1-dependent signaling requires a coreceptor, the low-density lipoprotein receptor–related proteins 5 and 6 (LRP). More recently, it has become clear that two additional unrelated, single-pass receptor tyrosine kinases, receptor tyrosine kinase-like orphan receptor 2 (ROR2) and receptor-like tyrosine kinase (RYK), can bind to Wnts. In contrast to signaling events downstream of the Frizzled/LRP receptors, the mechanisms of ROR2- and RYK-mediated Wnt signaling are poorly understood (Angers and Moon, 2009). RYK has been shown to bind to Frizzleds (Lu et al., 2004; Kim et al., 2008; Li et al., 2009). However, Frizzleds are not required for Wnt/RYK signaling in all contexts (Inoue et al., 2004; Schmitt et al., 2006; Harris and Beckendorf, 2007; Li et al., 2009), which suggests a distinct molecular mechanism of Wnt/RYK signaling.

RYK is required for CTNNB1-dependent Wnt signaling, as loss of function of RYK inhibits the ability of Wnt-3A to activate CTNNB1 signaling and that this activity depends on endogenous RYK. Conversely, in loss-of-function studies, both RYK and CTNNB1 are required for Wnt-3A–mediated activation of CTNNB1. Finally, we identify the Caenorhabditis elegans orthologue of MIB1 and demonstrate a genetic interaction between ceMIB and lin-18/RYK in vulva development. These findings provide insights into the mechanisms of Wnt/RYK signaling and point to novel targets for the modulation of Wnt signaling.
and neuronal differentiation of neocortical progenitor cells (Lyu et al., 2008).

RYK is also involved in stimulating CTNNB1-independent Wnt signaling pathways. Genetic and biochemical studies demonstrate that Wnt-5 and RYK cooperate to regulate axon pathfinding in vivo and in vitro in multiple species (Liu et al., 2005; Keeble et al., 2006; Wouda et al., 2008; Li et al., 2009; Miyashita et al., 2009). Moreover, Wnt-5 and Wnt-11 signal through RYK to regulate cell movements during convergent extension of zebrafish (Danio rerio) and frog (Xenopus laevis) embryos (Kim et al., 2008; Lin et al., 2010). Though not directly tested in these studies, Wnt-5 and Wnt-11 have been reported to signal independently of CTNNB1 in a context-dependent manner.

The molecular mechanisms of Wnt/RYK signaling remain elusive. RYK is composed of a leucine-rich extracellular domain with a WIF-type Wnt binding region, a single transmembrane domain, and an intracellular tyrosine kinase domain (Halford and Stacker, 2001). Most evidence suggests that RYK signaling does not depend on intrinsic kinase activity; however, RYK has been shown to interact with other kinases including Ephrin receptors and Src, which suggests cross-talk with other tyrosine kinase signaling cascades (Halford et al., 2000; Trivier and Ganesan, 2002; Kamitori et al., 2005; Wouda et al., 2008). In addition, in neuronal precursor cells, RYK is cleaved by γ-secretase, and treatment with Wnt-3A leads to the nuclear accumulation of the RYK intracellular domain (RYKICD; Lyu et al., 2008). Whether the RYKICD binds to DNA or has any role in the regulation of transcription remains an open question. Finally, two recent studies show that RYK is internalized in response to Wnt-5 and Wnt-11 (Kim et al., 2008; Lin et al., 2010). However, neither the consequences of RYK internalization nor the factors that regulate internalization are known.

In this paper, we identified RYK-associated proteins by affinity purification mass spectrometry and leveraged the results to investigate the mechanisms of Wnt/RYK signaling. Although this approach identified multiple proteins that co-purify with RYK, in this study we focused on the E3 ubiquitin ligase MIB1. Biochemical experiments revealed that MIB1 promotes the ubiquitination and turnover of full-length RYK and results in the loss of RYK expression at the cell surface. Moreover, using both gain- and loss-of-function experiments in vitro and in vivo, we found an as of yet unreported functional relationship between RYK and MIB1 in the positive regulation of Wnt/CTNNB1 signaling.

Results

Affinity purification mass spectrometry of RYK
To identify proteins that were candidates for mediating Wnt/RYK signaling, we used tandem affinity purification of RYK followed by mass spectrometry (Angers et al., 2006; Major et al., 2007). We stably expressed glue-tagged RYK at low levels in HEK293T cells, which have intact Wnt/RYK signaling (Lu et al., 2004). We isolated glue-tagged RYK from cells that were treated and lysed under several conditions to identify the maximum number of interaction partners (Table S3). Because RYK is known to be cleaved and internalized, we also performed affinity purification of glue-tagged RYKICD from stably expressing HEK293T cells. Using this combined approach, we identified several known RYK-interacting proteins including CDC37, ubiquitin, HSP90, and ephrin receptors (Fig. 1 A and Table S3; Halford et al., 2000; Trivier and Ganesan, 2002; Kamitori et al., 2005; Lyu et al., 2009).

We identified a total of 66 RYK-associated proteins that met the following criteria: (a) the proteins were identified in at least three of eight independent pull-down experiments, (b) the proteins were represented by at least two unique peptides, and (c) the proteins had not been previously identified as common nontarget contaminants (Gingras et al., 2005; Major et al., 2008).

Using bioinformatic analyses, we classified these proteins into several complexes or families of proteins involved in cell signaling, cell adhesion, cell cycle and apoptosis, actin remodeling, planar cell polarity, ubiquitination, and protein trafficking (Fig. 1 A and Table S4). Among the novel proteins identified, the most frequently observed and the highest in abundance was the E3 ubiquitin ligase MIB1.

RYK exists in a protein complex with MIB1
To confirm the finding that RYK and MIB1 coexist in a protein complex, we used affinity purification and Western blotting. We found that streptavidin purification of glue-tagged RYK, RYKICD, or of a plasma-membrane tethered, i.e., myristoylated, version of the RYKICD (MYR-RYKICD) efficiently coprecipitated endogenous MIB1 as compared with untransfected HEK293T cells or those expressing glue-tagged negative control proteins (Fig. 1, B and C). Moreover, in the reverse pull-down experiment, flag-tagged MIB1 coprecipitated glue-RYKICD (Fig. 1 D).

Previous studies have shown that substrates of MIB1-mediated ubiquitination bind to its N-terminal MIB1/HERC2-type zinc finger (Itoh et al., 2003). Therefore, we performed an analysis to define the domain of MIB1 required for forming a complex with RYK. We transiently expressed a series of flag-tagged N- and C-terminal truncations of MIB1 in cells stably transfected with glue-RYKICD. We found that the RYKICD efficiently coprecipitated with the N terminus of MIB1 (minimal binding domain aa 1–124) but not the C terminus (Fig. 1, B and D). We also observed that RYK could form a complex with a point mutant of MIB1 (C997S) that reduces its enzymatic activity (Fig. 1 D). In summary, these results show that RYK and MIB1 exist in a protein complex in a manner requiring the interaction of the ICD of RYK and the substrate recognition domain of MIB1.

MIB1 promotes the ubiquitination of RYK
MIB1 acts as an E3 ubiquitin ligase for several substrates (Jin et al., 2002; Itoh et al., 2003; Yoo et al., 2006; Zhang et al., 2007). Thus, we investigated whether MIB1 could promote the ubiquitination of RYK. We performed nickel affinity
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ubiquitin to other substrates (Jin et al., 2002; Itoh et al., 2003; Yoo et al., 2006; Zhang et al., 2007). Similarly, we found that MIB1 with a point mutation (C997S) in the third ring domain or MIB1 lacking all three ring domains (aa 1–729; MIB1-ΔRN3) significantly reduced or eliminated, respectively, the ability of MIB1 to promote the ubiquitination of RYK (Fig. 2 A).

Figure 1. Affinity purification mass spectrometry identifies MIB1 as a novel interaction partner with RYK. [A] Illustration depicts the RYK-associated protein interaction network discovered by affinity purification mass spectrometry (black lines). The STRING database was used to curate additional interactions among proteins found to interact with RYK (gray lines). The size of the node indicates the total number of peptides identified in eight independent purifications. [B] Schematic representation of RYK and MIB1 proteins depicting predicted domains. [C] Western blot for endogenous MIB1 demonstrates interaction with streptavidin-affinity-purified glue-tagged RYK, RYKICD, or a membrane tethered form of the RYKICD (MYR-RYKICD). Untransfected, glue-TCF4, and glue-NCK2 cells served as negative controls. [D] Western blot for glue-RYKICD demonstrates interactions with flag affinity-purified full-length MIB1, the N terminus of MIB1, or a point mutant (C997S) of MIB1 that renders it catalytically impaired. N-terminally truncated MIB1 and negative controls do not copurify RYK. Blots represent n = 3.
immunoreactivity of streptavidin affinity-purified glue-RYKICD (Fig. 2 B). In addition, a point mutation in MIB1 (S807A) that inhibits phosphorylation by PAR1 and subsequent auto-ubiquitination (Ossipova et al., 2009) did not interfere with the ubiquitination of RYK (Fig. 2 B). Thus, MIB1 is sufficient to promote the ubiquitination of RYK in the absence of exogenous ubiquitin.

Mouse Ryk (mRYK) shares 93% amino acid identity with human RYK and 98% identity within the ICD, including 18 of 19 lysines. mRYK is cleaved by $\gamma$-secretase and the

In addition, we found that MIB1 could efficiently promote the ubiquitination of glue-tagged RYKICD or MYR-RYKICD but not RYK lacking the ICD (RYKΔICD). Mutant MIB1, C997S, and truncated MIB1, ΔRN3, have little or no catalytic activity with respect to RYK. (B) Western blot analyzing streptavidin purification of glue-RYKICD. The blot shows the conjugation of endogenous ubiquitin promoted by MIB1 and by nonphosphorylatable MIB1-S807A, but not by MIB1-C997S. (C) Western blot analyzing metal affinity purification of His-tagged ubiquitin under denaturing conditions showing that MIB1 can promote the ubiquitination of C-terminal myc-tagged mouse RYK, RYKICD, and noncleavable RYK-RC. Blots represent $n > 3$. The asterisk indicates a nonspecific band.

Figure 2. MIB1 promotes RYK ubiquitination. (A) Western blot analyzing metal-affinity purification of His-tagged ubiquitin under denaturing conditions. The blot shows that MIB1 can promote the ubiquitination of the N-terminal glue-tagged RYK, RYKICD, and membrane-tethered MYR-RYKICD but not RYK lacking the ICD (RYKΔICD). Mutant MIB1, C997S, and truncated MIB1, ΔRN3, have little or no catalytic activity with respect to RYK. (B) Western blot analyzing streptavidin purification of glue-RYKICD. The blot shows the conjugation of endogenous ubiquitin promoted by MIB1 and by nonphosphorylatable MIB1-S807A, but not by MIB1-C997S. (C) Western blot analyzing metal affinity purification of His-tagged ubiquitin under denaturing conditions showing that MIB1 can promote the ubiquitination of C-terminal myc-tagged mouse RYK, RYKICD, and noncleavable RYK-RC. Blots represent $n > 3$. The asterisk indicates a nonspecific band.

We next investigated whether MIB1 could enhance the conjugation of endogenous ubiquitin to RYK. Cells transfected with MIB1 but not MIB1-C997S robustly increased the ubiquitin

immunoreactivity of streptavidin affinity-purified glue-RYKICD (Fig. 2 B). In addition, a point mutation in MIB1 (S807A) that inhibits phosphorylation by PAR1 and subsequent auto-ubiquitination (Ossipova et al., 2009) did not interfere with the ubiquitination of RYK (Fig. 2 B). Thus, MIB1 is sufficient to promote the ubiquitination of RYK in the absence of exogenous ubiquitin.

Mouse Ryk (mRYK) shares 93% amino acid identity with human RYK and 98% identity within the ICD, including 18 of 19 lysines. mRYK is cleaved by $\gamma$-secretase and the
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We next tested the hypothesis that MIB1 overexpression decreases Ryk protein levels. We found that the steady-state levels of full-length glue-Ryk were decreased by MIB1 in a dose-dependent manner (Fig. 3 A). In contrast, neither glue-RykICD nor the plasma membrane–tethered form, MYR-glue-RykICD, were significantly decreased by overexpression of MIB1 (Fig. 3 A). We also tested whether C-terminal myc-tagged mRyk levels were affected by MIB1. Consistent with our results with human Ryk, overexpression of MIB1 decreased the levels of full-length mRyk-myc but not of mRyk-ICD-myc (Fig. 3 B). Thus, MIB1 can promote the turnover of full-length Ryk but not the RykICD. We also discovered that the levels of mRyk-RC-myc were decreased by MIB1 (Fig. 3 B). This result suggested that γ-secretase cleavage is not required for the ability of MIB1 to degrade Ryk. Indeed, in a direct test of this hypothesis we found that MIB1 was able to degrade wild-type full-length mRyk-myc in the presence of γ-secretase inhibitors (Fig. 3 C). Collectively, these data demonstrate that the extracellular region of Ryk is required for efficient turnover and imply the presence of additional regulatory factors.

We next tested whether the inhibition of the proteasome or lysosome could restore Ryk levels in the presence of overexpressed MIB1. We found that addition of either MG132 or chloroquine, small molecule inhibitors of the proteasome and lysosome, respectively, resulted in increased steady-state levels of Ryk. Interestingly, the inhibition of the expression of TSG101, a member of the ESCRT pathway, reverses the ability of MIB1 to degrade Ryk. In summary, our results with human and mouse Ryk indicate that MIB1 is sufficient to promote the ubiquitination of Ryk.

**MIB1 promotes the turnover of Ryk**

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**Figure 3. MIB1 promotes Ryk protein turnover.** (A) Western blot of cell lysates showing that MIB1 cDNA overexpression results in a decrease in full-length Ryk but not RykICD or MYR-RykICD. (B) Western blot of cell lysates showing that MIB1 cDNA overexpression results in a decrease in full-length mouse Ryk and noncleavable Ryk-RC but not mouse RykICD. (C) Western blot of cell lysates showing that MIB1 cDNA overexpression results in a decrease in full-length mouse Ryk in the presence of γ-secretase inhibitors. (D) Western blot of cell lysates showing that MIB1-dependent degradation of Ryk can be reversed by inhibition of the proteasome with MG132 or inhibition of the lysosome with chloroquine. (E) Western blot of cell lysates demonstrating that the inhibition of the expression of TSG101, a member of the ESCRT pathway, reverses the ability of MIB1 to degrade Ryk. (F) Western blot of cell lysates demonstrating that the turnover of full-length Ryk in the presence of translation inhibition by cycloheximide can be reversed with proteasome or lysosome inhibition. (G) Western blot of cell lysates demonstrating that the turnover of full-length Ryk in the presence of cycloheximide can be reversed by knockdown of MIB1 with siRNAs. Blots represent n = 3.
of glue-RYK and inhibited the ability of MIB1 to degrade glue-RYK (Fig. 3 D). Trafficking of ubiquitinated cargo to the lysosome depends on members of the ESCRT pathway, such as TSG101 (Raiborg and Stenmark, 2009). Therefore, to further test whether MIB1 overexpression leads to lysosomal degradation of RYK, we assessed levels of glue-RYK in the context of TSG101 inhibition by siRNA. We found that TSG101 siRNA transfection increased steady-state levels of RYK and inhibited the ability of MIB1 to degrade RYK (Fig. 3 E). Collectively these results show that MIB1 overexpression is sufficient to promote the degradation of RYK by the proteasome and the lysosome.

The findings in Fig. 3 imply that endogenous MIB1 could regulate the rate of RYK turnover. To test whether endogenous MIB1 is required for RYK degradation, we first monitored the expression of full-length glue-RYK after inhibition of protein translation with cycloheximide. We found that RYK levels decreased within 2 h of cycloheximide treatment (Fig. 3 F). Moreover, we found that we could delay the rate of RYK turnover by simultaneously treating cells with proteasome or lysosome inhibitors consistent with ongoing RYK turnover by these organelles (Fig. 3 F).

Next, we transfected cells with control or MIB1 siRNAs and evaluated RYK turnover. Importantly, we found that the degradation of RYK in cycloheximide-treated cells was delayed by MIB1 loss of function (Fig. 3 G). These results support a model in which endogenous MIB1 promotes the turnover of full-length RYK through ubiquitination and degradation.

**MIB1 colocalizes with RYK on Rab5-positive intracellular membranes**

We also investigated the subcellular localization of RYK and MIB1. When glue-RYK was expressed in either HEK293T or Hela cells, it localized to the plasma membrane and intracellular...
vesicles (Fig. 4 A and Fig. S2 A). However, when RYK was coexpressed with flag-MIB1, both proteins colocalized on large intracellular membrane and vesicular organelles (Fig. 4, B and E; and Fig. S2 B). Notably, these organelles were also positive for the endocytic marker Rab5 (Fig. 4 B; Sorkin and von Zastrow, 2009).

Because MIB1 ubiquitinates RYK, we also asked whether the enzymatic activity of MIB1 was required for the relocation of RYK. Co-expression of MIB1-C997S with RYK resulted in colocalization in protein aggregates or aggregated vesicles near the plasma membrane and in intracellular vesicles (Fig. 4, C and E; and Fig. S2 C). As with wild-type MIB1, these structures were also positive for Rab5. Although MIB1-C997S has reduced enzymatic activity, residual ubiquitination of RYK (Fig. 2 A) could account for this change in RYK localization. Thus, we also tested the enzyme-dead MIB1, MIB1-ΔRN3. Unlike MIB1 or MIB1-C997S, MIB1-ΔRN3 had no effect on RYK localization, though both proteins colocalized at sites along the plasma membrane (Fig. 4, D and E; and Fig. S2 D).

**MIB1 quantitatively reduces RYK surface expression in the absence of protein degradation**

We next used an independent method to quantify surface RYK expression on individual cells. As we had found that MIB1 regulates RYK protein turnover, MIB1-mediated decreases in total RYK might account for the loss of surface expression of RYK. Moreover, although the images were scored blind, immunocytochemistry is inherently qualitative and subjective. Therefore, we used flow cytometry to quantify the intensity of RYK surface expression in the context of proteasome and lysosome inhibition. HEK293T cells expressing glue-RYK were treated with chloroquine and MG132 for 2 h and then fixed in paraformaldehyde. We then used immunofluorescence under nonpermeabilizing conditions to detect the N-terminal glue epitope of RYK (Fig. 5 A). Using this method, we found that overexpression of MIB1 decreased the surface expression of RYK by >40% as compared with the vector-only control (Fig. 5, B and C). Expression of MIB1-C997S and MIB1-ΔRN3 had little effect on RYK surface expression (Fig. 5, B and C). Moreover, as predicted from the fact that MIB1 does not ubiquitinate RYKΔICD (Fig. 2 A), we found that MIB1 overexpression did not change RYKΔICD surface expression compared with the vector-only control (Fig. 5 D).

We also tested whether endogenous MIB1 regulates surface expression of RYK. As expected, the inhibition of MIB1 expression by siRNA transfection increased surface RYK expression (Fig. 5, E and F). Collectively, these results suggest that MIB1 is both necessary and sufficient for the expression of RYK at the plasma membrane.

**RYK and MIB1 are required for Wnt/CTNNB1 signaling**

Because RYK is required for Wnt/CTNNB1 signaling (Lu et al., 2004), we hypothesized that MIB1 might also regulate Wnt signaling. We measured the activity of a luciferase-based transcriptional reporter (CTNNB1-activated reporter or Wnt/β-catenin–activated luciferase reporter [BAR]; Biechele et al., 2009) in HEK293T cells after transfection of siRNAs against RYK or MIB1. Consistent with previously published data (Lu et al., 2004), we found that three independent siRNAs targeting RYK (Fig. S1, A and C) inhibited Wnt-3A–dependent activation of BAR by >50% (Fig. 6 A). Importantly, we also found that three independent siRNAs targeting MIB1 (Fig. S1, B and D) inhibited activation of BAR by Wnt-3A to a similar degree as RYK siRNAs (Fig. 6 A).

In addition, we investigated whether RYK and MIB1 loss of function could reduce the expression of endogenous Wnt/CTNNB1 target genes. We found that treating HEK293T cells with Wnt-3A induced a modest increase in the levels of AXIN2 that could be partially reversed by MIB1 or RYK loss of function (Fig. 6 B). Unpublished results from our laboratory show that the osteosarcoma cell line, U2OS, has a more robust transcriptional response to Wnt-3A than HEK293T cells (unpublished data). Therefore, we also tested whether MIB1 and RYK were required for Wnt/CTNNB1 signaling in U2OS cells. First, we confirmed that siRNAs targeting either RYK or MIB1 were able to reduce Wnt-3A–mediated activation of BAR in U2OS cells (Fig. 6 C). Likewise, we found that Wnt-3A increased levels of AXIN2 transcript more than fourfold in the context of control siRNAs and that this effect was significantly reduced by siRNAs targeting either RYK or MIB1 (Fig. 6 D). The expression of another Wnt-target gene, NKD1, was also reduced by RYK and MIB1 loss of function in U2OS cells (Fig. 6 E). Finally, we confirmed this effect in a third cell line. As with HEK293T and U2OS, HeLa cells also showed reduced responses to expression of the Wnt-3A–induced target genes AXIN2, NKD1, and TNRFSF19 after RYK or MIB1 loss of function (Figs. 6 F and S2, E and F). In summary, these results demonstrate that endogenous RYK and MIB1 are required for Wnt/CTNNB1-dependent transcription in multiple cell lines.

**MIB1 and RYK act upstream of CTNNB1 stabilization**

Our data suggest that MIB1 regulates RYK by inhibiting its localization at the cell surface. Thus, RYK and MIB1 loss of function could inhibit activation of Wnt/CTNNB1 signaling by affecting other components of the signaling pathway that operate near the plasma membrane. We found that siRNAs targeting either RYK or MIB1 inhibited the activation of BAR in cells overexpressing Wnt-3A or Disheveled 3 but had little effect on BAR activation by CTNNB1 or stabilized mutant CTNNB1 (CTNNB1*, S33A, S37A, T41A, and S45A; Fig. 7 A). Thus, RYK and MIB1 likely regulate events that occur early in Wnt pathway activation.

To further investigate the temporal requirement for MIB1 in Wnt signaling, we performed a time-course analysis of HEK293T cells treated with Wnt-3A. We found that cells transfected with MIB1 siRNA show reduced levels of phospho-LRP6, an early marker of Wnt activation, within 30 min of Wnt-3A treatment (Fig. 7 B; Davidson et al., 2005; Zeng et al., 2005). MIB1 loss of function inhibited the ability of Wnt-3A to reduce serine/threonine phosphorylation of CTNNB1; however, we did not see robust changes in Wnt-3A–induced steady-state levels of total CTNNB1 with MIB1 siRNA (Fig. 7 B).
Collectively, our data suggest that RYK and MIB1 act together in a complex to regulate Wnt/CTNNB1 signaling. To directly test this model, we first asked whether MIB1 overexpression was sufficient to activate Wnt signaling. Indeed, overexpression of MIB1 activated BAR in a dose-dependent manner in the presence or absence of Wnt-3A (Fig. 8A). Moreover, we found that MIB1 was sufficient to induce AXIN2 transcription, the phosphorylation of LRP6, and stabilization of CTNNB1 (Figs. 8, B and C). Second, if MIB1 and RYK act in a protein complex, then MIB1 might require interaction with RYK to activate Wnt/CTNNB1 signaling. We found that, unlike full-length MIB1, N-terminally truncated MIB1 mutants, e.g., aa 125–1,007, which do not form a complex with RYK (Fig. 1 C), cannot activate BAR (Fig. 8D). Moreover, we found that MIB1 mutants, which can form a complex with

HEK293T cells have high levels of membrane-associated CTNNB1, and levels of CTNNB1 are not appreciably changed by Wnt-3A treatment. Therefore, we also blotted for CTNNB1 in the lysates that had been stripped of junctional CTNNB1 using conA-sepharose resin (Miller and Moon, 1997). Using this approach, we found that RYK and to a lesser degree MIB1 siRNAs reduced the ability of Wnt-3A to increase the “signaling pool” of CTNNB1 after 1 h of treatment with Wnt-3A (Fig. 7C). Like our results in HEK293T cells, we found that the transfection of RYK or MIB1 siRNAs reduced the ability of Wnt-3A to increase LRP6 phosphorylation, reduce CTNNB1 phosphorylation, and stabilize CTNNB1 in U2OS cells (Fig. 7D). Collectively, our data show that RYK and MIB1 are required for Wnt/CTNNB1 signaling through regulation of protein phosphorylation upstream of CTNNB1 stabilization.

MIB1 activates Wnt/CTNNB1 signaling in an RYK-dependent manner

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...resulting in a bivulva phenotype. Thus, we asked whether MIB1 was also important in this process. First, we performed BLASTp analysis to identify the C. elegans homologue of human MIB1. We identified the protein, Q9U2C5, in the UniProt database. Using reverse BLASTp analysis, we found that Q9U2C5 has equal identity with human MIB1 and MIB2 (23%), which is consistent with the existence of a single ancestral protein (Fig. 9A). Similar to human MIB1 and MIB2, Q9U2C5 contains seven predicted Ankyrin repeats and two C-terminal ring fingers. The gene encoding Q9U2C5, Y47D3A.22, has predicted genetic interactions with several members of the Notch pathway, including crb-1/Crumbs, glp-1/Notch, F10D7.5/Neuralized, and lin-12/Notch, which suggests conservation of function with human MIB1 and MIB2 in Delta/Notch signaling (Zhong and Sternberg, 2006). Therefore, we reasoned that Q9U2C5/Y47D3A.22, henceforth ceMIB, could be a functional homologue of human MIB1.

We next turned to loss of function in vivo to assess whether ceMIB was also important in this process. First, we performed BLASTp analysis to identify the C. elegans homologue of human MIB1. We identified the protein, Q9U2C5, in the UniProt database. Using reverse BLASTp analysis, we found that Q9U2C5 has equal identity with human MIB1 and MIB2 (23%), which is consistent with the existence of a single ancestral protein (Fig. 9A). Similar to human MIB1 and MIB2, Q9U2C5 contains seven predicted Ankyrin repeats and two C-terminal ring fingers. The gene encoding Q9U2C5, Y47D3A.22, has predicted genetic interactions with several members of the Notch pathway, including crb-1/Crumbs, glp-1/Notch, F10D7.5/Neuralized, and lin-12/Notch, which suggests conservation of function with human MIB1 and MIB2 in Delta/Notch signaling (Zhong and Sternberg, 2006). Therefore, we reasoned that Q9U2C5/Y47D3A.22, henceforth ceMIB, could be a functional homologue of human MIB1.

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C. elegans MIB1 RNAi genetically interacts with lin-18/RYK

Wnt signaling is required for vulva cell fate determination in C. elegans (Inoue et al., 2004). Mutations of lin-18/RYK disrupt the oriented division of p7.p vulval precursor cells, ultimately resulting in a bivulva phenotype.
MIB1 overexpression activates Wnt/CTNNB1 signaling in an RYK-dependent manner and that endogenous RYK and MIB1 were required for the Wnt-dependent activation of Wnt/CTNNB1 signaling. Finally, we discovered that \textit{lin-18}/RYK and \textit{ceMIB} show a genetic interaction in \textit{C. elegans} vulva development. These findings raise several interesting questions surrounding the relationship of RYK and MIB1 to Wnt signaling.

Does MIB1 regulate RYK trafficking in response to Wnt? Recent studies demonstrate that RYK undergoes endocytosis in response to Wnt binding in vivo (Kim et al., 2008; Lin et al., 2010). We found that MIB1 regulated surface expression of RYK. However, we were unable to detect changes in the surface localization of RYK after treatment with Wnt-3A in our system (unpublished data). Thus, we could not test whether MIB1 was required for Wnt-induced changes in RYK surface expression. Nevertheless, it remains formally possible that MIB1 regulates the endocytosis of RYK after ligand binding.

What is the significance of MIB1-associated RYK ubiquitination? The ubiquitination of transmembrane proteins can lead to endocytosis and trafficking to a variety of intracellular...

Discussion

Our goal was to investigate novel mechanisms involved in the regulation of Wnt/RYK signaling. We identified MIB1 as a novel protein interaction partner with RYK. We found that overexpressed RYK and MIB1 colocalized and that the overexpression of MIB1 leads to the loss of surface RYK expression. In addition, biochemical studies revealed that MIB1 promotes the ubiquitination and degradation of RYK. We found that MIB1 overexpression activates Wnt/CTNNB1 signaling in an RYK-dependent manner and that endogenous RYK and MIB1 were required for the Wnt-dependent activation of Wnt/CTNNB1 signaling. Finally, we discovered that \textit{lin-18}/RYK and \textit{ceMIB} show a genetic interaction in \textit{C. elegans} vulva development. These findings raise several interesting questions surrounding the relationship of RYK and MIB1 to Wnt signaling.

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MIB1 is required for Wnt/RYK signaling

MIB1 ubiquitination of RYK signals the endocytosis of a multimeric complex composed of RYK and other Wnt receptors. The endocytosis of LRP6 and Frizzled has been shown to be required for efficient activation of Wnt/CTNNB1 signaling (Yamamoto et al., 2006; Yamamoto et al., 2008). Consistent with this model, we found that the knockdown of RYK or MIB1 leads to reduced LRP6 phosphorylation and levels in response to Wnt. Whether MIB1 directly regulates the kinases necessary for LRP6 phosphorylation is unknown.

A second possibility is that MIB1 acts downstream of RYK. In this model, the ubiquitination/degradation of RYK could reflect a negative feedback loop regulating RYK surface expression. The kinase MARK (microtubule affinity regulating kinase)/PAR1 can phosphorylate and modulate the function of MIB1 (Ossipova et al., 2009). PAR1 is also known to bind the membrane-associated Wnt pathway protein Disheveled (Sun et al., 2001). Thus, Wnt signaling through RYK could modulate PAR1 phosphorylation of MIB1 and its subsequent activity on...
an unknown effector of Wnt signaling. Further studies are needed to assess the biochemical relationships of MIB1, RYK, and other members of the Wnt pathway, and the requirement of MIB1 and RYK for Wnt-mediated changes in protein complexes, posttranslational modification, and localization should help clarify these issues.

In conclusion, we demonstrated the first evidence that the ubiquitin ligase MIB1 acts to regulate Wnt/CTNNB1 signaling in a complex with the Wnt receptor RYK. MIB1 is an important regulator of multiple developmental phenomena, many of which are known to require Wnt signaling. Moreover, because MIB1 acts in other signal transduction pathways, such as Delta/Notch, it represents a novel node for mitigating pathway cross-talk. In the future, it will be important evaluate developmental and disease phenotypes resulting from MIB1 mutation or deletion in the context of both Notch and Wnt signaling.

Materials and methods

Cell culture, transfections, and drugs

Hela, HEK293T, and U2OS cells were obtained from the American Type Culture Collection and cultured in DME plus 10% FBS and 1% penicillin-streptomycin (Invitrogen). siRNA transfections were performed with Lipofectamine 2000 (Invitrogen). siRNAs were used singly or in equimolar pools at 20 nM total concentration. siRNA sequences are given in Table S1. cDNA transfections were performed with Lipofectamine 2000 (HEK293T; Invitrogen) or FuGene HD (HeLa; Roche). For protein turnover studies, cells were treated overnight with 10 µM DAPT or 1 µM L-685,485 (HEK293T; Invitrogen) or Fugene HD (HeLa; Roche). For protein turnover studies, cells were treated overnight with 10 µM DAPT or 1 µM L-685,485 (HEK293T; Invitrogen) or Fugene HD (HeLa; Roche).

C. elegans maintenance and RNAi

Wild-type(N2) and lin-18(e620) strains were gifts from the Priess laboratory (Fred Hutchinson Cancer Research Center, Seattle, WA). To identify the C. elegans homologue of MIB1, we performed BLASTp analyses using the tools at the National Center for Biotechnology Information and UniProt databases. Phylogenetic analysis was performed with ClustalW2 using the neighbor joining method. Full-length Y47D3A.22/ceMIB was amplified from a pool of cDNA from mixed stage wild-type [N2] worms and cloned into the L4440 vector for RNAi feeding. To minimize the maternal contribution, worms were fed on RNAi plates for more than two generations before scoring. Live L4 stage larvae were mounted in PBS on glass slides. Differential interference contrast images were collected at room temperature using NIS Elements software and a Plan Fluor 40×/0.90 NA objective lens on an inverted wide-field microscope (TE; Nikon). Scores represent the sum of three individual experiments.

Molecular cloning

RYK was amplified by PCR [GenBank/EMBL/DBJ] accession no. NM_001005861, base pairs 229–1923] from human fetal cDNA and cloned into a modified pGLUE vector (Angers et al., 2006) using Ascl and NotI. The signal sequence from CHRNA7 was added 5’ to the streptavidin-binding peptide in the glue tag 5’-ATGGCGTGCAGCCCCGGCGGCTGTG-GCTGGCGCTGGGCCGCCTCTCCTGCTCCTGACGTAGCCTG-3’. RYK lacking the ICD (base pairs 229–882) was amplified by PCR and cloned into signal sequence pGLUE using Ascl and NotI. The ICD of RYK was amplified by PCR (base pairs 883–1923) and cloned into pGLUE using Ascl and NotI. MYR-GLUE-RYKICD was made by appending the myristoylation sequence of SRC 5’-ATGGTGACAAAAAGAGCAGAACAAAGCATTTAG-3’ 5’ to the streptavidin-binding peptide in pGLUE-RYKICD. MYR-RYK was received from W. Lu (University of Southern California, Los Angeles, CA). All other cDNA clones were generated by standard PCR-based cloning. Whole-plasmid PCR was used for site-directed mutagenesis of MIB1. All clones were verified by sequence analysis of the open reading frames.

Quantitative RT-PCR

RNA was extracted using the RNAeasy RNA extraction kit as per the manufacturer’s recommendations [QIAGEN]. First-strand cDNA was synthesized using oligodeoxythymidine and random hexamer primers with the Revert-aid kit (Fermentas). Quantitative RT-PCR was performed on a LightCycler 480 using 10 µl reactions (Roche). Primers used are listed in Table S2.

Immunocytochemistry, immunoprecipitation, and Western blotting

For immunocytochemistry, cells were seeded onto poly-l-lysine (Sigma-Aldrich)-coated coverslips and fixed in 4% paraformaldehyde in PBS at 22°C for 10 min. For overexpression studies, cells were transfected with 100 ng/cm² of plasmid 18–20 h before fixation. Secondary antibodies
were donkey or goat anti-rat, anti-mouse, or anti-rabbit Alexa Fluor 488, 568, or 647 conjugates [Invitrogen]. Cells were counter-stained with DAPI and mounted in Prolong Gold [Invitrogen]. Images were collected at room temperature with NIS Elements software on a microscope (Eclipse Ti; Nikon) coupled to a Nikon A1R confocal operating in Galvano mode and using a Plan-Apochromat 60x/1.40 NA objective lens [Nikon]. Quantification was performed by an observer that was blinded to the treatments. Images were processed for publication using GIMP 2.6.8 and Inkscape 0.47.

For immunoprecipitation and Western blotting, cells were lysed in 50 mM Tris, pH 8.0, 1% Triton X-100, 0.1% SDS, 0.2% sodium deoxycholate, 150 mM sodium chloride, and 2 mM EDTA plus complete protease and phosphatase inhibitors [Roche]. Affinity purification was performed for 2–4 h at 4°C with streptavidin-Sepharose [GE Healthcare] or anti-flag M2 affinity gel [Sigma-Aldrich]. For metal affinity purification cells, were lysed in 6 M urea, 300 mM sodium chloride, and 10 mM imidazole in 100 mM sodium phosphate buffer, pH 7.4, and purified 2–4 h at 22°C with Ni-NTA agarose [Qiagen]. The His-tagged ubiquitin plasmid was a gift from D. Bohmann [University of Rochester, Rochester, NY].

Primary antibodies used were CTNNB1 [9561 and 9562; Cell Signaling Technology], β-tubulin [17816; Sigma-Aldrich], flag [F3165 and F7425; Sigma-Aldrich], HA [HA10, 1867423; Roche], LRP6 [2568 and 3395; Cell Signaling Technology], MB1 [M5948; Sigma-Aldrich], Rab5 [SC-28570; Santa Cruz Biotechnology, Inc.], TSG101 [Ab83; Abcam], and ubiquitin [P4D1, SC-8017; Santa Cruz Biotechnology, Inc.].

Surface labeling and flow cytometric analysis Cells were treated for 2 h with 100 µM chloroquine and 10 µM MG132, harvested by trituration, and fixed for 10 min in 1% paraformaldehyde in PBS. N-terminally tagged proteins were detected with an HA antibody for 2 h at room temperature followed by donkey anti-rabbit Alexa Fluor 488 for 30 min under nonpermeabilization conditions. At least 10,000 events per sample were collected on a FACS Canto II flow cytometer [BD] and analyzed with FlowJo [Tree Star].

Affinity purification mass spectrometry and bioinformatics Tandem affinity purification was performed essentially as described previously [Angers et al., 2006; Major et al., 2007; Major et al., 2008]. In brief, cell lysates from 15 × 15 cm plates of HEK293T cells were subjected to the Search Tool for the Retrieval of Inter-Network datasets are provided in Table S3. After compiling and filtering the data, proteins were submitted to the Search Tool for the Retrieval of Inter-acting Genes/Proteins (STRING) database to curate known interactions. Annotation of proteomics hits. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201107021/DC1.

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