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Frodo Links Dishevelled to the p120-Catenin/Kaiso Pathway: Distinct Catenin Subfamilies Promote Wnt Signals

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Summary

p120-catenin is an Arm repeat protein that interacts with varied components such as cadherin, small G proteins, kinases, and the Kaiso transcriptional repressor. Despite recent advances in understanding the roles that p120-catenin and Kaiso play in downstream modulation of Wnt/β-catenin signaling, the identity of the upstream regulators of the p120-catenin/Kaiso pathway have remained unclear. Here, we find that p120-catenin binds Frodo, which itself interacts with the Wnt pathway protein Dishevelled (Dsh). In *Xenopus laevis*, we demonstrate that Wnt signals result in Frodo-mediated stabilization of p120-catenin, which, in turn, promotes Kaiso sequestration or removal from the nucleus. Our results point to Dsh and Frodo as upstream regulators of the p120-catenin/Kaiso signaling pathway. Importantly, this suggests that Wnt signals acting through Dsh regulate the stability of p120-catenin in addition to that of β-catenin, and that each catenin promotes its respective signal in parallel to regulate distinct, as well as shared, direct downstream gene targets.

Introduction

Canonical Wnt signals are mediated through β-catenin/TCF/LEF (Wnt/β-catenin pathway), whereas Wnt/PCP signals are mediated through Rho GTPases or other intermediates. Each pathway and their interplay are fundamental to a wide array of developmental processes as well as tumor progression (Logan and Nusse, 2004; Moon et al., 2004). Wnt/β-catenin pathway activation leads to β-catenin's metabolic stabilization and generally involves multiple components such as Wnt extracellular ligand, transmembrane Fz (Frizzled) receptor and LRP (Low-Density Lipoprotein-Related) coreceptor, and intracellular Dsh (Dishevelled). Stabilized β-catenin enters the nucleus and interacts with DNA-binding transcriptional repressors of the TCF/LEF (hereafter called the TCF) family, resulting in the activation (relief of repression) of downstream target genes (Logan and Nusse, 2004; Moon et al., 2004). β-catenin's signaling activity is further modulated by other cytoplasmic determinants (Gottardi and Gumbiner, 2004) and by a variety of nuclear cofactors (Logan and Nusse, 2004).

Catenins (excepting α-catenin) contain central Armadillo (Arm) repeats facilitating direct association with cadherin cell-cell adhesion proteins, with modulators or components of the cytoskeleton, and with assorted other polypeptides such as transcriptional repressors, involved in developmental and pathologic gene regulation. Catenin family proteins include the well-studied β-catenin, its close homolog plakoglobin (γ-catenin), and the similar but not as closely related p120-catenin subfamily members. Catenins participate in an array of processes spanning the areas of cell adhesion, migration and cytoskeletal function, survival and proliferation, and cell differentiation and apoptosis (Anastasiadis and Reynolds, 2000; Gottardi and Gumbiner, 2001; Hatzfeld, 2005; Reynolds and Roczniak-Ferguson, 2004; van Roy and McCrea, 2005).

p120-catenin was first characterized as a favored Src and receptor tyrosine kinase substrate (Reynolds et al., 1992, 1994), and it later became recognized as the founding member of the p120 subclass that additionally includes ARVCF, δ-catenin, and p0071 (Anastasiadis and Reynolds, 2000). While both β-catenin and p120-catenin bind cadherin cytoplasmic domains, p120-catenin subfamily members associate with the more membrane-proximal region implicated in cadherin dimerization and adhesive/motility functions as well as in cadherin endocytosis and metabolic stability (Davis et al., 2003; Reynolds and Roczniak-Ferguson, 2004). p120-catenin has additionally been found bound to kinesin and microtubules (Franz and Ridley, 2004; Yanagisawa et al., 2004), and considerable attention has also been focused on its key roles in modulating Rho GTPases and, thereby, cytoskeletal and cell cycle functions (Anastasiadis et al., 2000; Anastasiadis and Reynolds, 2001; Ohkubo and Ozawa, 1999; Yanagisawa et al., 2004). Also, tissue-specific knockout studies of p120-catenin in mice, as well as knockdown approaches in *Xenopus*, have supported p120-catenin's essential developmental roles in cadherin and Rho GTPase modulation (Ciesiolka et al., 2004; Davis and Reynolds, 2006; Elia et al., 2006; Fang et al., 2004; Perez-Moreno et al., 2006).

Further insights into p120-catenin's developmental functions have come from studies of its direct involvement in relieving Kaiso-mediated gene repression (Kim et al., 2004; Park et al., 2005). While less well understood than β-catenin-directed activation of Wnt gene targets (through relief of TCF/LEF-mediated repression) (Daniels and Weis, 2005), p120-catenin relieves Kaiso-mediated repression of genes likewise active within developmental and pathologic contexts (Kim et al., 2004; Park et al., 2005; Spring et al., 2005). Indeed, in certain cases, the Kaiso repressor has been shown to bind Kaiso consensus sites within promoter regions also harboring functional TCF sites (Wnt/β-catenin gene targets). In such

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cases, coordinate relief of Kaiso and TCF repression is thought to occur through the respective actions of p120- and β-catenin. The mechanism of β-catenin relief of TCF-mediated repression appears to be different from that of p120-catenin-directed relief of Kaiso, in which the latter repressor is thought to be displaced from promoter DNA upon p120-catenin association (Kim et al., 2004; Park et al., 2005; Spring et al., 2005). Despite such distinctions, it appears that limited parallels may be drawn between the β-catenin/TCF and p120-catenin/Kaiso pathways and their shared downstream actions on certain gene targets.

Kaiso was first identified in association with p120-catenin (Daniel and Reynolds, 1999), and it belongs to the BTB/POZ (Broad complex, Tramtrak, Bric à brac/POx virus and Zinc finger) family of proteins (Bardwell and Treisman, 1994). BTB/POZ proteins contain an N-terminal POZ domain that engages in protein-protein interactions (dimerization and corepressor interactions) and a C-terminal (C_2H_2) zinc finger domain responsible for DNA association (Albagli et al., 1995; Bardwell and Treisman, 1994). Among the BTB/POZ proteins, Kaiso appears to be unique in possessing dual specificity, recognizing both sequence-specific consensus sites and methylated CpG dinucleotides (Daniel et al., 2002; Prokhortchouk et al., 2001; Ruzov et al., 2004; Yoon et al., 2003).

Recently, it was reported that p120-catenin and Kaiso modulate sequence-specific gene targets that include *Wnt-11*, *Siamois*, *Matrilysin*, *Rapsyn*, and *S1004A* (Defossez et al., 2005; Kim et al., 2004; Park et al., 2005; Rodova et al., 2004; Ruzov et al., 2004; Spring et al., 2005; Yoon et al., 2003). As noted, a number of these genes are also directly responsive to Wnt/β-catenin signals (Gumbiner, 2005; Kim et al., 2004; Park et al., 2005). Thus, while likely to regulate distinct gene targets in many cases, it appears that numerous coregulated genes exist where parallel p120- and β-catenin pathways converge within the nucleus. An important remaining question that we have addressed in this study regards the mechanism of upstream p120-catenin/Kaiso pathway regulation, which, for example, might be initiated at the plasma membrane in response to ligand:receptor interactions.

Frodo (Functional regulator of Dishevelled in ontogenesis) and the closely related Dapper were identified by virtue of their functional and physical interactions with the central Wnt pathway component Dishevelled (Dsh) (Cheyette et al., 2002; Gloy et al., 2002). While each remains incompletely understood, Frodo/Dapper positively or negatively modulate the Wnt pathway, thus participating in multiple developmental processes such as axis induction, neural development, and morphogenic movements (Brott and Sokol, 2005; Cheyette et al., 2002; Gloy et al., 2002; Hikasa and Sokol, 2004; Waxman, 2005). Here, we report that Frodo associates with and stabilizes the p120-catenin protein, enabling p120-catenin to more effectively relieve Kaiso-mediated repression. To our knowledge, our findings support a new function of Frodo, and thereby Dsh and other upstream Wnt signaling components including Wnt ligands, in regulating p120-catenin activity within the p120-catenin/Kaiso pathway. It thus appears that the Wnt/β-catenin and p120-catenin/Kaiso pathways converge at both upstream and downstream points, suggesting considerable functional integration of distinct catenin subfamily members in development and, conceivably, disease.

Results

Identification of p120-Catenin as a Frodo-Interacting Partner

With the intent of examining Frodo developmental mechanisms, yeast two-hybrid assays were performed by using Frodo as "bait" in conjunction with a *Xenopus* gastrula cDNA expression library (Itoh et al., 2000). Resulting positive clones included the carboxy-terminal half of xp120-catenin, beginning with the sixth repeat of the central Armadillo domain. This potential interaction was consistent with the partially shared cytoplasmic and nuclear subcellular distributions of Frodo and p120-catenin (Aho et al., 2002; Brott and Sokol, 2005).

To test for the existence of Frodo:p120-catenin interactions in vivo, we performed coimmunoprecipitation from *Xenopus laevis* embryo extracts by using anti-Frodo antibody. This endogenous assay clearly supported the coassociation of Frodo and p120-catenin (Figure 1A, lane 3). The reverse endogenous precipitation of p120-catenin, followed by western blot detection of Frodo, was more difficult to interpret due to the appearance of background signals (data not shown). In a partial exogenous expression context, HA-tagged Frodo (HA-Frodo) mRNA was injected into cleavage-stage embryos and extracts (obtained at stages 11–12) employed in coimmunoprecipitation tests. Similar to endogenous Frodo:xp120-catenin coassociation, overexpressed HA-Frodo coprecipitated endogenous xp120-catenin (Figure 1B). Because p120-catenin additionally binds the Kaiso transcriptional repressor to relieve Kaiso-dependent gene repression (Kelly et al., 2004b; Kim et al., 2004; Park et al., 2005; Spring et al., 2005), we tested whether Frodo is also able to associate with Kaiso. Coinjection of Myc-Frodo mRNA with either HA-xp120-catenin or HA-xKaiso mRNA and subsequent coimmunoprecipitation showed that while p120-catenin interacts with Frodo as expected based on our above-described tests, Kaiso repressor does not (Figure 1C, compare lanes 4 and 6). Next, to characterize Frodo's interaction domain with p120, we generated a series of Frodo deletion constructs (a–e) (Figure 1D). Unlike the previously mapped interaction of Frodo with Dsh (Gloy et al., 2002), the carboxy-terminal PDZ-binding motif of Frodo is not required for the Frodo:p120-catenin interaction. Rather, the middle region (189–807 amino acids) of Frodo appears to be the primary region permitting direct or indirect association with p120-catenin (Figures 1D and 1E). Collectively, these data suggest that Frodo interacts with p120-catenin in vivo (endogenous or exogenous expression contexts) and in vitro (yeast two-hybrid).

Frodo Regulates Kaiso-Dependent Transcriptional Repression

Several reports have proposed that p120-catenin acts as a regulator of the Kaiso transcriptional repressor (Kim et al., 2004; Park et al., 2005; Spring et al., 2005; van Roy and McCrea, 2005). Based on the association

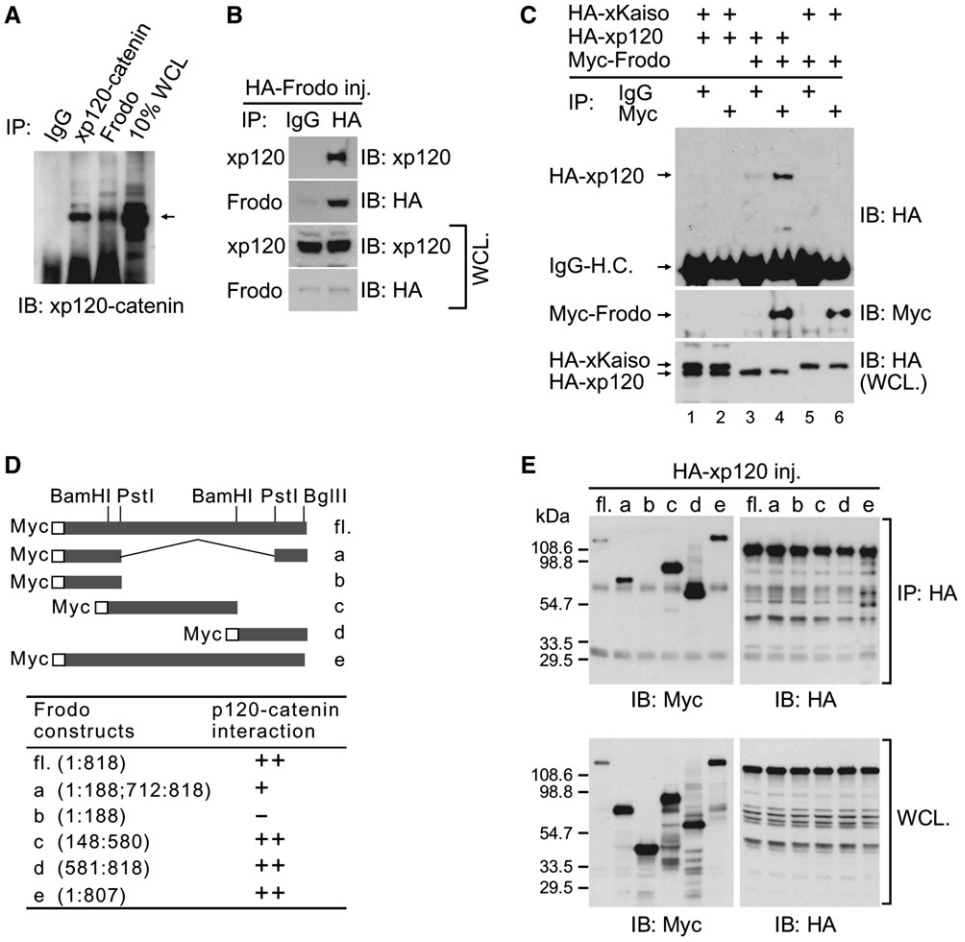

Figure 1. Interaction between p120-Catenin and Frodo

(A) Uninjected embryos were lysed at stages 10–11 and immunoprecipitated with anti-xp120-catenin or anti-Frodo antibody, followed by immunoblotting with anti-p120-catenin polyclonal antibody.

(B) HA-Frodo mRNA was injected into each blastomere of two-cell embryos (0.25 ng to each blastomere). Anti-HA immunoprecipitates were subjected to immunoblotting with anti-xp120-catenin antibody. WCL (whole cell/embryo lysates) served as loading controls.

(C) Either HA-xKaiso or HA-xp120-catenin mRNA (0.25 ng, positive control) was coinjected with Myc-Frodo mRNA (0.25 ng) into both blastomeres at the two-cell stage. Anti-Myc immunoprecipitates were immunoblotted with anti-HA antibody and did not resolve a Frodo:Kaiso complex (lane 6).

(D) Frodo cDNA deletion constructs (a–e). The table indicates the relative association of Frodo deletion constructs with p120-catenin shown in (E).

(E) Frodo deletion constructs (a–e) were coexpressed with HA-xp120-catenin (in vitro-transcribed mRNAs coinjected into one blastomere of two-cell embryos). Gastrula (stage 12) embryo extracts were subjected to coimmunoprecipitation with IgG (negative control) or anti-HA antibody. Bottom blots indicate overexpressed Frodo deletion constructs and xp120-catenin in WCL.

of Frodo with xp120-catenin, we wished to examine the impact of Frodo on Kaiso-mediated repression, and thereby the possible involvement of Frodo in the newly discovered p120-catenin/Kaiso signaling pathway. Since it was demonstrated that Kaiso suppresses *xWnt-11* transcription by direct Kaiso:DNA association (Kim et al., 2004), we first tested whether Frodo overexpression (Frodo mRNA injection) or Frodo depletion (Frodo-morpholino [Frodo-MO] injection) alters *xWnt-11* transcript levels. Employing semiquantitative RT-PCR (reverse transcriptase-polymerase chain reaction), it was observed that Frodo depletion reduces endogenous *xWnt-11* transcription and, conversely, that Frodo overexpression raises *xWnt-11* transcription (Figure 2A). To verify the specificity of Frodo's effects, we used xWnt-11 reporter plasmids harboring KCS (Kaiso consensus sequence; wild-type KCS, CTGCAA; mutant KCS, CTTCAA [the altered base is underlined]) (Kim et al., 2004). As expected, while Frodo depletion by Frodo-MO reduced wild-type KCS-xWnt-11 promoter activity, it had no effect on the mutant KCS-xWnt-11 promoter construct (Figure 2B). These data suggest that Frodo specifically contributes to relieving Kaiso-mediated gene repression.

Owing to the established role of p120-catenin in relieving Kaiso-mediated gene repression, we wished to test if Frodo's effects on Kaiso transcriptional activity might occur through p120-catenin. We therefore tested the dependency of Frodo's effects on p120-catenin by coinjecting Frodo mRNA with p120-catenin-morpholino (p120-MO), and we evaluated the impact on *xWnt-11* mRNA transcript levels by using semiquantitative RT-PCR of embryo extracts. As shown in Figure 2C (compare lanes 2 and 4), the activation of *xWnt-11* mRNA

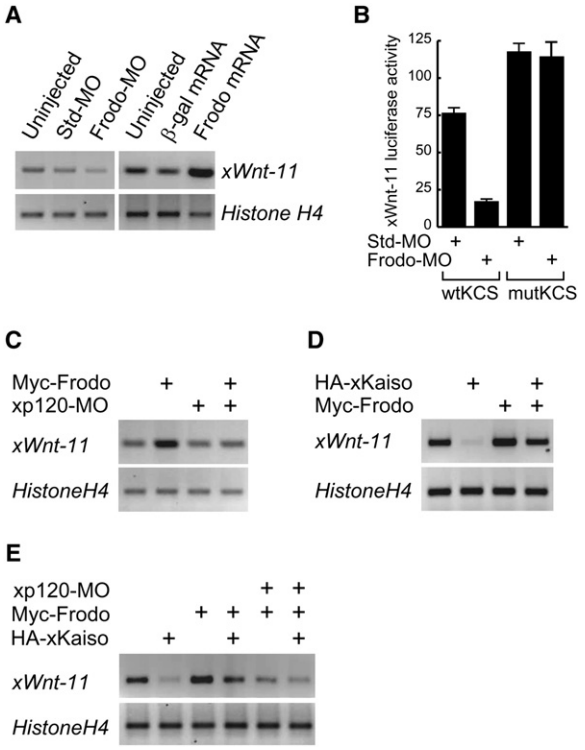

Figure 2. Frodo Regulation of the xKaiso Target Gene, *xWnt-11*

(A) Frodo-morpholino (Frodo-MO, 20 ng) or standard-morpholino (Std-MO, 20 ng) was injected into both blastomeres at the two-cell stage (lanes 1–3). Semiquantitative RT-PCR was performed to detect *xWnt-11* and *Histone H4* transcripts. Alternatively, either HA-Frodo mRNA (0.5 ng) or β-galactosidase (β-gal) mRNA (0.5 ng) was injected for subsequent semiquantitative RT-PCR (lanes 4–6).

(B) Wild-type (WT) versus mutant KCS (mutKCS) *xWnt-11* luciferase reporter plasmid (0.5 ng) was coinjected with Frodo-MO (20 ng). At stage 12, eight embryos per condition were collected to quantitate luciferase activity.

(C) Either Myc-Frodo mRNA (0.5 ng) or xp120-catenin-morpholino (xp120-MO, 20 ng) were coinjected into both blastomeres at the two-cell stage. To normalize either morpholino or mRNA concentration across samples, Std-MO or β-gal mRNA was added. The embryos were harvested at stage 11 for semiquantitative RT-PCR.

(D) Either HA-xKaiso (0.5 ng) or Myc-Frodo (0.5 ng) was injected into one-cell-stage embryos, followed by RNA extraction at stage 11.

(E) Either HA-xKaiso (0.5 ng) or Myc-Frodo (0.5 ng) was coinjected with xp120-catenin-morpholino (10 ng) into both blastomeres of embryos at the two-cell stage. Total amounts of either RNA or morpholino delivered were normalized with GFP mRNA or Std-MO, respectively. Embryos were collected and processed at stages 11–12, and RT-PCR was used to asses effects on xWnt-11 transcription. All semiquantitative RT-PCR was repeated more than three times with similar outcomes.

Error bars represent the standard deviation from three independent experiments.

by Frodo overexpression was inhibited by p120-MO injection. Moreover, the derepression of *xWnt-11* by Frodo overexpression is partially diminished by Kaiso overexpression (Figure 2D, compare lanes 3 and 4). Because such exogenous Kaiso only modestly counteracted Frodo-induced activation of *xWnt-11*, we asked if endogenous p120-catenin might have lowered Kaiso's impact. We therefore depleted endogenous xp120-catenin through the coinjection of xp120-catenin-morpholino (along with Frodo and Kaiso mRNAs) and assessed *xWnt-11* activation. As expected, upon the depletion of xp120-catenin, exogenous Kaiso exhibited a more pronounced inhibition of Frodo-induced *xWnt-11* activation (Figure 2E, compare lanes 4 and 6). These results imply that Kaiso-mediated repression of gene targets is subject to modulation by Frodo together with p120-catenin, presumably the result of Frodo:p120-catenin association.

Regulation of Kaiso:Promoter Association by Frodo

It is thought that p120-catenin relieves Kaiso-mediated transcriptional repression by dissociating Kaiso from gene control regions and/or by cytoplasmically sequestering Kaiso, the latter perhaps via the masking of Kaiso's NLS (nuclear localization signal) (Kim et al., 2004; Park et al., 2005; Spring et al., 2005). However, unknown at present are the detailed molecular mechanism(s) of p120-catenin's downstream functions, or until this report, the identity of upstream stimuli or molecules that direct p120-catenin's actions. To address Frodo's impact on Kaiso:DNA association, we applied in vivo chromatin immunoprecipitation (ChIP) assays (Kim et al., 2004; Park et al., 2005). Although faint due to the in vivo (whole-embryo) nature of the experiment, semiquantitative-ChIP-PCR repeatedly indicated Myc-xKaiso association with the gene promoter of xWnt-11 as well as *Siamois* (Figure 3A, compare lanes 1 and 2). Consistent with previous studies (Kelly et al., 2004a; Kim et al., 2002; Park et al., 2005; Spring et al., 2005), p120-catenin overexpression with Myc-Kaiso reduced the latter's binding to its promoter targets (Figure 3A, compare lanes 2 and 8), confirming the inhibitory role of p120-catenin in Kaiso:DNA interactions. While slightly less pronounced, Frodo overexpression interestingly also reduced Myc-xKaiso's association with target promoters (Figure 3A, compare lanes 2 and 5), suggesting that Frodo likewise inhibits Kaiso:DNA association.

We next asked if diminished Kaiso:DNA association occurring after p120-catenin overexpression resulted from the subcellular redistribution of Kaiso in response to Frodo. For this purpose, we employed immunofluorescent staining of HeLa cells transiently transfected with HA-Frodo, Myc-xKaiso, and/or HA-xp120-catenin. As shown in Figure 3B, Kaiso is localized principally to the nucleus; however, upon p120-catenin cotransfection, Kaiso is dramatically relocalized to the cytoplasm. Interestingly, Frodo-cotransfected cells also exhibited efficient relocalization (Figure 3B, right panel). Since Frodo induces the nuclear export of Kaiso in the apparent absence of a biochemical Frodo:Kaiso interaction (see Figure 1C, lane 6), we suspected that Frodo-induced relocalization of Kaiso might be mediated by the initial relocalization of p120-catenin by Frodo. Indeed, upon Frodo overexpression, p120-catenin's dispersed pattern became perinuclear and overlapped with that of Frodo (Figure S1; see the Supplemental Data available with this article online). It thus appears that Frodo is capable of altering p120-catenin localization, with p120-catenin in turn sequestering Kaiso away from the nucleus (gene promoters) to the cytoplasm.

Frodo Rescues Kaiso-Induced Gastrulation Defects

Previously, we demonstrated that Kaiso-mediated modulation of *xWnt-11* gene activity is essential for proper convergent extension during early gastrulation (Kim

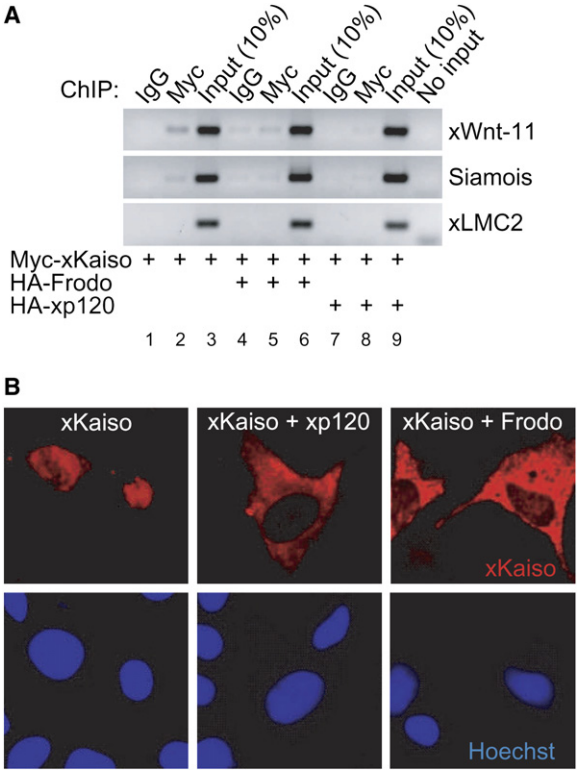

Figure 3. Inhibition of Kaiso:DNA Association by Frodo

(A) Myc-xKaiso mRNA was coinjected with HA-Frodo or HA-xp120-catenin mRNA into one-cell embryos (0.5 ng each mRNA). At stage 12, the embryos were processed for ChIP (chromatin immunoprecipitation) and semiquantitative PCR by using primers situated on either side of established KCS within *xWnt-11* or *Siamois* promoter sequences. The *XLMC2* promoter sequence served as a negative control.

(B) HeLa cells were transiently transfected with Myc-xKaiso, HA-xp120-catenin, or HA-Frodo as indicated. A total of 24 hr after transfection, cells were fixed, incubated with anti-Myc antibody, and then visualized by using Texas red-conjugated anti-mouse antibody and fluorescence microscopy. Hoechst 33258 was used for nuclear staining.

et al., 2004; Park et al., 2005). We therefore reasoned that Frodo may also affect convergent extension through p120-catenin-mediated regulation of the Kaiso repressor. Based on our molecular and biochemical evidence that Frodo reduces Kaiso's repression of gene targets (see Figures 2 and 3), we tested for in vivo effects of Frodo on Kaiso in *Xenopus* early embryogenesis/gastrulation. As expected, Kaiso overexpression resulted in gastrulation defects evident in incomplete blastopore closure (Figure 4B) and defects in convergent extension (data not shown) (Park et al., 2005). However, the coinjection of Frodo mRNA with Kaiso mRNA resulted in the partial (~30%) rescue of such gastrulation failure phenotypes, and this occurred in a dose-dependent manner (Figures 4C–4E). Additionally, to test whether endogenous Frodo is required for normal gastrulation, we depleted it through Frodo-MO injection. Although the injection of smaller amounts of Frodo-MO did not result in notable effects, higher doses (20 ng) produced severe gastrulation failures in a large proportion (~72%) of embryos relative to controls (Figure 4G). Our data thus support an essential role of Frodo in early embryogenesis in keeping with earlier reports (Gloy et al., 2002). Also assessed was Frodo overexpression, which in a dose-dependent manner produced gastrulation failures in a proportion of embryos (Figure 4F). We expect that Frodo's overexpression effects resulted in part from the activation (relief of repression) of largely unknown Kaiso repressor gene targets; however, one known transcriptional target to consider was xWnt-11 (Kim et al., 2004).

To test if Frodo overexpression phenotypes could be rescued by reducing Wnt/PCP signaling downstream of xWnt-11, we employed established Dsh-ΔPDZ and Dsh-ΔDEP constructs (Kim et al., 2004; Wallingford et al., 2000). Since gastrulation failures resulting from the derepression of *xWnt-11* by Kaiso depletion had been partially rescued by Dsh-ΔDEP (Kim et al., 2004), we anticipated it might likewise reduce overexpression effects of Frodo on Wnt/PCP signaling. Indeed, to a partial (~20%) extent, Dsh-ΔDEP repeatedly rescued the Frodo overexpression phenotype (see Figure 4H). This result was consistent with our previous finding showing that Dsh-ΔDEP (but not Dsh-ΔPDZ) could partially rescue the xWnt-11 activation phenotype after Kaiso depletion (Kim et al., 2004). Moreover, we found that the overexpression of dominant-negative DN-xWnt-11, which produces convergent-extension failures (Heisenberg et al., 2000), was fractionally rescued by Frodo mRNA microinjection; such an effect presumably arose in part from increased endogenous xWnt-11 expression (data not shown). Our whole-embryo rescue data (Figures 4C–4E and 4H) support models wherein Frodo acts to relieve downstream Kaiso gene repression of developmentally important (e.g., *xWnt-11*) genes modulating Wnt/PCP signaling.

Frodo Regulates p120-Catenin Protein Stability

Because p120-catenin modulates cadherin function, we tested if, by association, Frodo has an impact on cell-cell adhesion. One could imagine, for example, that Frodo's effects on adhesion might be coordinated with those that relieve Kaiso-mediated repression of gene targets in the nucleus. We assessed Frodo-overexpressing effects by using standard *Xenopus* naive ectoderm (ex vivo animal cap) cell reaggregation assays. Frodo overexpression, however, did not obviously alter cell reaggregation, suggesting that cadherin-mediated cell-cell adhesion is not responsive to Frodo (Figure S2). A caveat of course is that mild effects may have escaped detection ex vivo but remain significant in vivo.

To further examine Frodo:p120-catenin interactions, but at the metabolic level, we tested whether Frodo depletion produces measurable changes in the level of p120-catenin within embryos. Indeed, Frodo depletion (Frodo-MO) resulted in significantly decreased p120-catenin levels, while, in contrast, the amount of Kaiso protein remained unchanged (Figure 5A, compare lanes 1 and 3 with lanes 2 and 4). As would be expected for the specificity of such a response, Frodo-MO-induced loss of p120-catenin occurred in a dose-dependent manner (data not shown). To further verify Frodo-MO (Frodo depletion) specificity in reducing p120-catenin levels, we performed rescue experiments in which Frodo-MO and Myc-Frodo mRNA were coinjected. As anticipated,

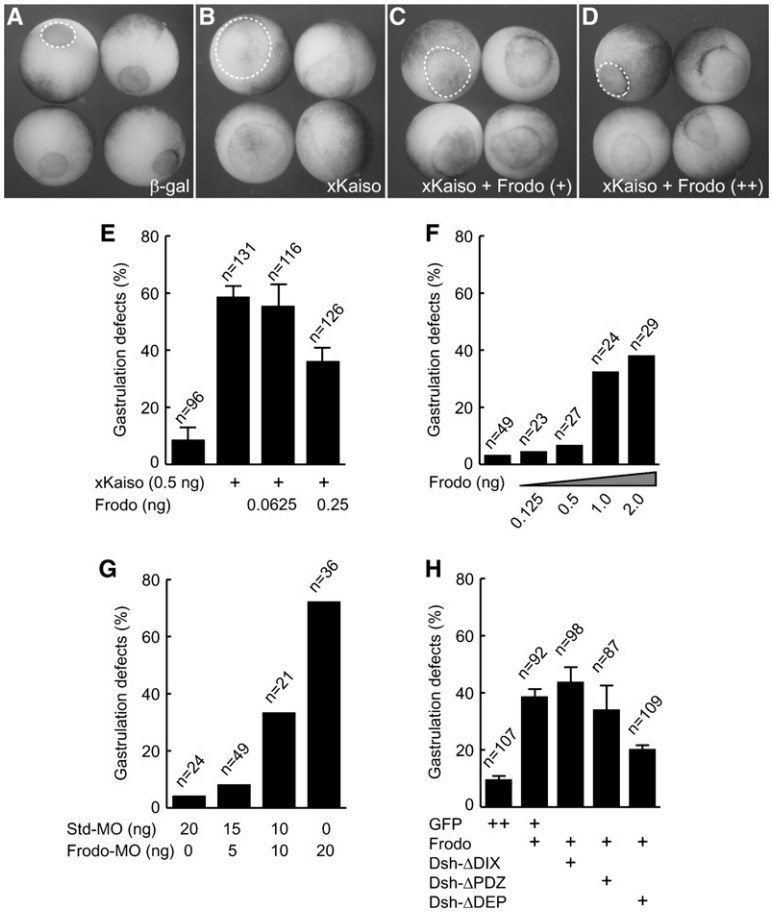

Figure 4. Frodo Partially Rescues Kaiso-Induced Gastrulation Failures

(A–D) xKaiso mRNA (0.5 ng) was coinjected with Frodo mRNA (0.0625/"+" ng or 0.25/"++" ng) into one-cell embryos (total mRNA normalized to 0.75 ng with β-gal mRNA). Gastrulation defects were visually scored at stages 10–12. Among three independent experiments, representative vegetal-pole images are displayed (dashed lines indicate blastopores).

(E) Quantitation of gastrulation-failure phenotypes.

(F) Prior to conducting rescue experiments, the impact of delivering varying doses of Frodo mRNA into one-cell embryos was determined.

(G) Increasing doses of Frodo-MO versus Std-MO were injected into one blastomere at the two-cell stage, and embryos were scored for defects at late gastrulation (stage 12.5).

(H) Frodo mRNA (2.0 ng) was coinjected with Dsh-ΔDIX, Dsh-ΔPDZ, or Dsh-ΔDEP into one blastomere of two-cell-stage embryos, and gastrulation-rescue effects were scored (250 pg each; GFP serving as the negative control).

Error bars represent the standard deviation from three independent experiments.

exogenous Frodo expression significantly (if not completely) recovered endogenous p120-catenin levels in a dose-dependent manner (Figure 5B). To widen our understanding of Frodo-like proteins in modulating p120-catenin protein levels, we also tested the effect of injecting a Dapper-morpholino (Dapper-MO). Dapper is a member of the Frd (Frodo/Dapper) protein family, and, similar to Frodo, interacts with Dsh protein (Cheyette et al., 2002). Although Frodo's and Dapper's roles in Wnt signaling are incompletely understood and may each include both positive and negative roles (Cheyette et al., 2002; Gloy et al., 2002), we observed that the depletion of either Frodo or Dapper in embryos resulted in decreased p120-catenin protein levels, with the Frodo-MO having the greater effect (Figure 5C). These results imply that Frodo and Dapper may have a common role in regulating p120-catenin protein levels.

Since p120-catenin's association with Kaiso acts to relieve Kaiso-dependent transcriptional repression (Kelly et al., 2004a; Kim et al., 2004; Park et al., 2005; Spring et al., 2005), alterations in p120-catenin protein levels in response to Frodo would be expected to have an impact on Kaiso gene targets (Figures 2 and 3). To test if p120-catenin:Kaiso association responds to Frodo levels, we coinjected Frodo mRNA versus Frodo-MO together with Myc-xp120-catenin and HA-xKaiso mRNAs (three-component injection). Subsequently, extracts from gastrula-stage (stage 11) embryos were assessed for HA-xp120-catenin:Myc-xKaiso association. Those embryos overexpressing Frodo (as opposed to β-galactosidase) demonstrated a reproducible increase in p120-catenin:Kaiso interactions (Figure 5D, compare lanes 6 and 8). Conversely, Frodo depletion (Frodo-MO) resulted in diminished p120-catenin:Kaiso associations (Figure 5D, compare lanes 2 and 4), apparently due to lowered levels of p120-catenin protein (Figure 5D, compare lanes 2 and 4 within the xp120-catenin western blot). Alternatively, when concanavalin A was used to precipitate glycoprotein complexes (such as cadherin), both the cadherin- and residual cytosolic-enriched fractions showed partial but reproducible increases in p120-catenin levels in response to Frodo overexpression (Figure 5E, lanes 2 and 4). These and the above-mentioned data suggest that Frodo positively affects p120-catenin protein levels, relieving Kaiso-mediated gene repression.

Wnt Signaling Is Involved in Frodo-Dependent p120-Catenin Protein Stabilization

Given that Frodo was initially identified as a Dsh-interacting protein in the Wnt/β-catenin signaling pathway (Gloy et al., 2002), we reasoned that Dsh might play a role in Frodo-mediated regulation of p120-catenin protein levels. Overexpression of Dsh resulted in modest but reproducible increases in p120-catenin protein as assessed through band-density quantification of ECL/western blots (Figure 6A). We then additionally evaluated the impact of established Dsh mutants (Dsh-ΔDIX, ΔPDZ, and ΔDEP) on p120-catenin protein levels, since these constructs have somewhat differential

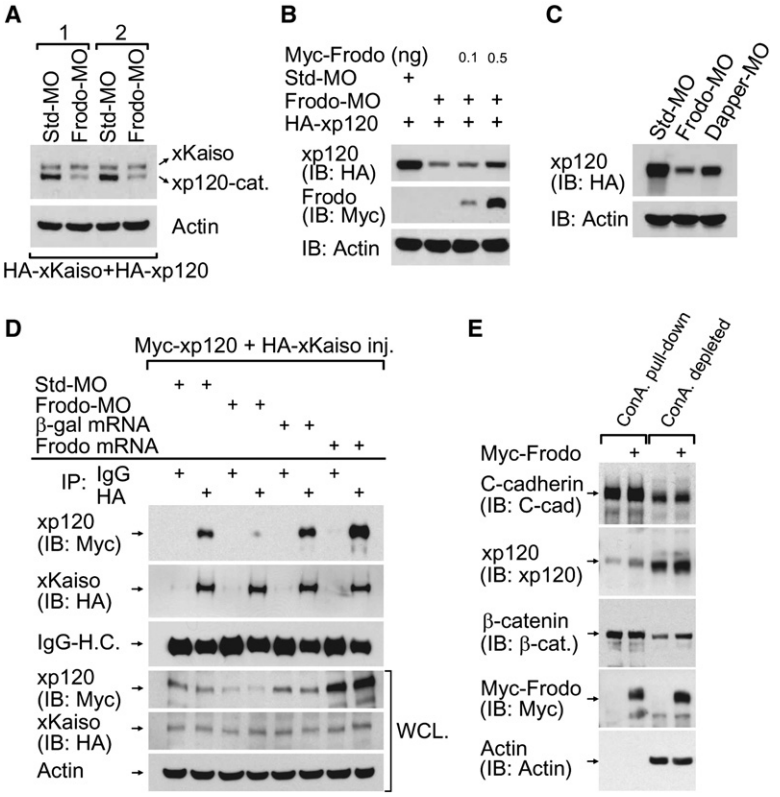

Figure 5. Frodo Depletion Destabilizes p120-Catenin Protein while Frodo Overexpression Stabilizes p120

(A) Either HA-xKaiso mRNA (0.25 ng) or HA-xp120-catenin mRNA (0.25 ng) and morpholino (10 ng Frodo-MO or Std-MO) were coinjected into both cells of two-cell embryos. Embryos were harvested at stages 11–12 for immunoblotting with anti-HA antibody. Actin was used as an internal loading control from whole cell/embryo extracts. The brackets marked "1" and "2" indicate results from two independent sets of embryos.

(B) Increasing amounts of Myc-Frodo mRNA (0.1 and 0.5 ng) were coinjected with Frodo-MO and HA-xp120-catenin mRNA (0.25 ng) into one blastomere at the two-cell stage. Embryos were harvested for immunoblot analysis of Myc-Frodo-generated rescues at stages 11–12.

(C) HA-xp120-catenin mRNA (0.25 ng) was coinjected with Frodo-MO (10 ng) or Dapper-MO (10 ng) into one blastomere at the two-cell stage, followed by immunoblot assessment of xp120-catenin levels in embryo extracts (stage 12).

(D) Morpholino (10 ng of Std-MO or Frodo-MO; lanes 1–4) or mRNA (0.25 ng of β-gal or Frodo mRNA) were coinjected with both Myc-xp120-catenin and HA-xKaiso mRNA (0.2 ng each) into one blastomeres at the two-cell stage. Embryos were collected at stage 11 (40 embryos per condition) and were used for immunoprecipitation/immunoblot analysis to assess Myc-xp120-catenin:HA-xKaiso association (using anti-HA antibody). WCL indicated equivalent expression of Myc-xp120-catenin as well as HA-xKaiso protein.

(E) Myc-Frodo mRNA (0.5 ng) was injected into the animal region of both blastomeres at the two-cell stage. At stages 10–11, embryos were lysed and incubated with concavanalin A (Con-A) beads for 30 min to precipitate cadherin-associated (glycosylated) protein complexes. After brief centrifugation, the pellet (ConA. pull-down fraction) and the supernatant (ConA. depleted fraction) were applied as equal fractions and subjected to SDS-PAGE/immunoblotting.

effects on Wnt/β-catenin versus Wnt/PCP pathways. Interestingly, Dsh-ΔDIX expression resulted in decreased p120-catenin presence (Figure 6B, lane 2). Given the Dsh-DIX domain's role in transducing Wnt/β-catenin signals, and in associating with Frodo (Gloy et al., 2002; Rothbacher et al., 2000), these data imply that Dsh:Frodo interactions modulate cellular p120-catenin levels. Next, to assess Dsh and Frodo epistasis in regulating p120-catenin, we tested whether lowered p120-catenin levels seen after Dsh-ΔDIX expression could be rescued by Frodo overexpression. Since Dsh and perhaps Frodo have scaffolding functions, a caveat is that such functional epistasis results would not necessarily reflect clear distinctions in the sequence of the components' associations. Nonetheless, as shown in Figure 6C, Frodo overexpression suppresses the effect of Dsh-ΔDIX in reducing p120-catenin protein levels (compare lanes 2 and 4), suggesting that Frodo is functionally downstream of Dsh.

One of the remaining puzzles is the means by which native Dsh and Frodo produce a positive effect on p120-catenin protein levels. Based on current models of how β-catenin protein levels are regulated via Dsh and a multicomponent destruction complex (Logan and Nusse, 2004; Moon et al., 2004), we hypothesized that p120-catenin may also undergo proteasome-mediated degradation. Indeed, when incubated with the 26S proteasome inhibitor MG132 (50 μM), p120-catenin levels were no longer reduced in embryos expressing Dsh-ΔDIX (Figure 6D). Analogously, while not graphic, p120-catenin metabolic turnover was at least partially reduced in Frodo-depleted embryos incubated in the presence of MG132 (Figure 6E).

Because GSK3β has a role in regulating β-catenin protein stability in response to Wnt signals (Liu et al., 2002; Polakis, 2002), we investigated its possible involvement with regard to p120-catenin. Endogenous p120-catenin levels were increased in a dose-dependent manner in mammalian MCF-7 and HeLa cell lines treated with up to 25 and 50 mM LiCl, a GSK3β inhibitor (Klein and Melton, 1996) (Figure 6F). At higher LiCl doses (100 mM), decreased p120-catenin levels were observed, perhaps resulting from cytotoxicity. Based on the participation of Dsh and Frodo in regulating p120-catenin protein levels/stability (also see Figures 5A, 6A, and 6B), we wished to test if the Wnt ligand itself affected p120-catenin protein levels. To activate the Wnt/β-catenin signaling pathway, we expressed xWnt-8 (activates Wnt/β-catenin) versus xWnt-5a (activates Wnt/PCP) in early embryos and assessed their respective effect on p120-catenin protein.

Embryonic overexpression of xWnt-8, but not xWnt-5a, resulted in modest increases in xp120-catenin protein levels, which were reproducible, although less than those observed (and expected) for β-catenin (Figure 6G). In contrast, in a mammalian cell line context, Wnt-3A

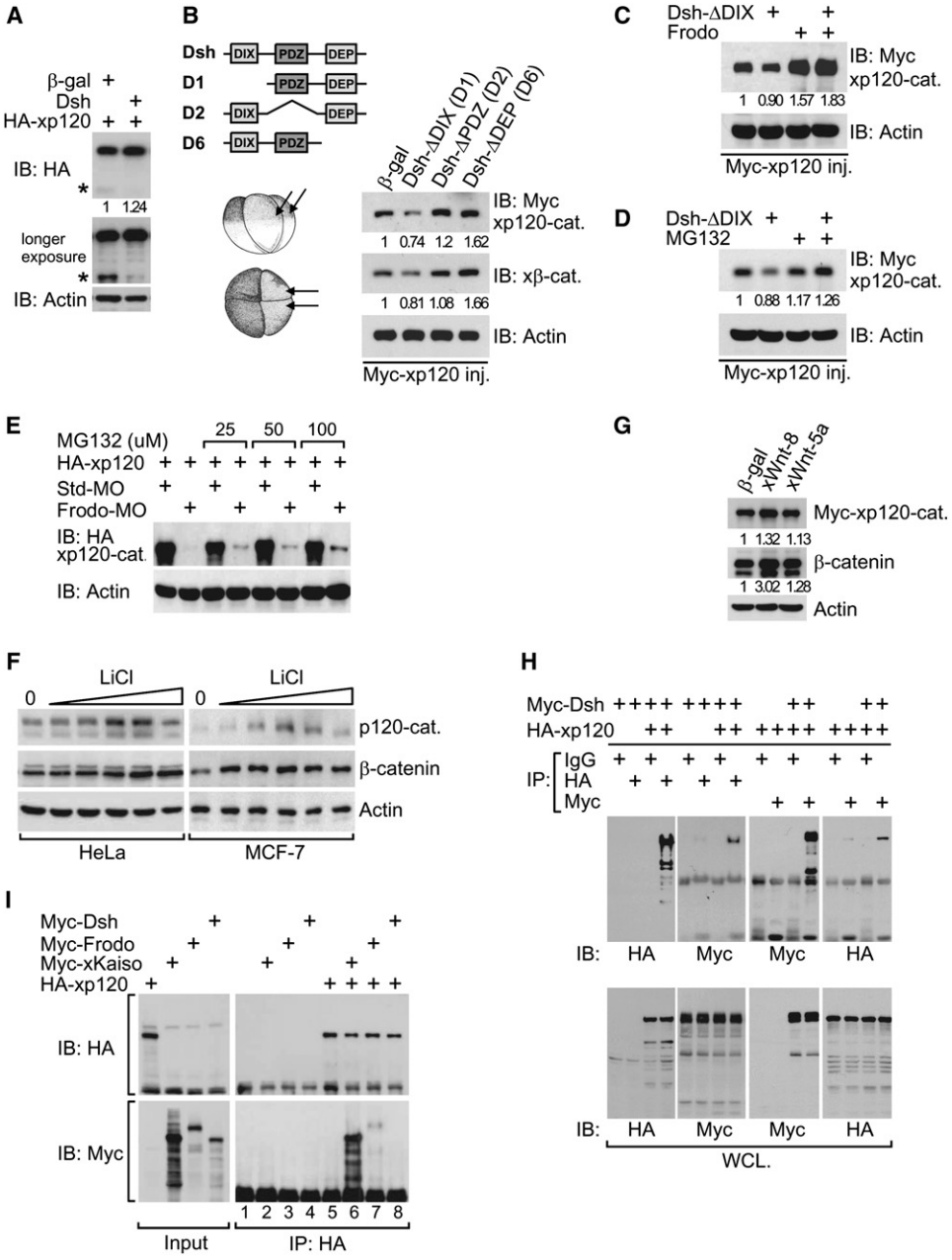

Figure 6. Dsh Regulates p120-Catenin Protein Stability

(A) Myc-Dsh mRNA (0.5 ng) was coinjected with HA-xp120-catenin mRNA (0.25 ng) into both blastomeres at the two-cell stage, and gastrulating embryos (stage 12) were collected for immunoblot analysis. The asterisk indicates what is likely to be a proteolytic degradation product of HA-xp120-catenin that is reproducibly less apparent upon Myc-Dsh cooverexpression. Quantitation of the relative band density of HA-xp120-catenin is indicated.

(B) A total of 0.5 ng Dsh deletion mutant mRNAs (ΔDIX, ΔPDZ, and ΔDEP) were utilized for coinjection with Myc-xp120-catenin mRNA (0.25 ng) into the dorsal animal region of two blastomeres (arrows indicate injection area) at the four-cell stage. At stage 12, embryos were harvested and lysates were immunoblotted with anti-Myc antibody. The β-catenin immunoblot served as a positive control for Dsh deletion mutant effects.

(C) Frodo abrogates the effect of Dsh-ΔDIX on p120-catenin protein stability. Each mRNA (0.5 ng Frodo and/or Dsh-ΔDIX) was coinjected with Myc-xp120-catenin mRNA (0.5 ng) into two blastomeres at the four-cell stage. Embryos were harvested at stage 12, and the level of Myc-xp120-catenin protein was assessed by immunoblot.

(D) Either Myc-xp120-catenin mRNA (0.5 ng) or Dsh-ΔDIX mRNA (0.5 ng) was injected into both blastomeres at the two-cell stage, followed by immediate treatment with MG132 (50 μM) or DMSO (negative control). Embryos were collected at stages 11–12 for subsequent immunoblot with anti-Myc antibody to detect Myc-xp120-catenin.

(E) Embryos injected with HA-xp120-catenin mRNA (0.5 ng) and with either Std-MO or Frodo-MO (20 ng) (coinjections into one blastomere at the two-cell stage) were immediately treated with varying concentrations of MG132 and later harvested at stages 11–12 for immunoblotting.

(F) MCF-7 mammalian cells were treated with the GSK3β inhibitor LiCl (5, 10, 25, 50, 100 mM) for 24 hr. Endogenous p120-catenin protein levels were analyzed by immunoblotting for p120-catenin. β-catenin protein served as a positive control for LiCl treatment.

(G) Increased p120-catenin protein by Wnt ligand. Either xWnt-8 or xWnt-5a mRNA (0.5 ng) was coinjected with HA-xp120-catenin mRNA into both blastomeres at the two-cell stage. At stage 12, the embryos were collected for further immunoblotting with anti-HA antibody.

(Wnt isolated from the conditioned media of L cells) failed to increase endogenous p120-catenin protein within HEK293 monolayers (data not shown). Thus, while not evident in all experimental systems, our in vivo (embryo) data suggest that an upstream Wnt ligand (xWnt-8) has the capacity to modestly raise p120-catenin protein levels.

Since the xWnt-8 induced increase in p120-catenin protein levels would be expected to promote p120's capacity to sequester Kaiso away from gene promoters, we tested the effects of xWnt-8 on Kaiso:promoter-DNA association by using ChIP assays. As expected, xWnt-8 expression lessened Kaiso binding to two of its known target gene promoters, xWnt-11 and Siamois (Figure S3, compare lanes 2 and 5).

Finally, at the biochemical level, we employed immunoprecipitation and in vitro binding assays to test if Dsh directly associates with p120-catenin (Figures 6H and 6I). While the expected direct interaction of p120-catenin with Frodo (see Figure 6I, lane 7), as well as that of p120-catenin with Kaiso (lane 6), was resolved, a direct interaction between Dsh and p120-catenin was not supported. Together, our results indicate that p120-catenin is metabolically stabilized in response to Wnt ligands through the involvement of Dsh and Frodo. Further, our data are consistent with the possibility that GSK3β-mediated phosphorylation of p120-catenin may modulate p120-catenin's (proteasomal and/or other) degradation/stability, and thereby its signaling capacity within the recently described p120-catenin/Kaiso developmental pathway.

Discussion

Frodo: Upstream Modulator of the p120-Catenin/Kaiso Signaling Module

Since p120-catenin associates with cadherin membrane-proximal domains in manners responsive to phosphorylation (Ohkubo and Ozawa, 1999; Skoudy et al., 1996; Thoreson et al., 2000), it is conceivable that plasma membrane- or cytoplasmically associated kinases or phosphatases have an impact on the entry of p120-catenin into a signaling/nuclear pool involving Kaiso. However, even in normal cells, p120-catenin is often not solely localized to cadherin-enriched cell-cell borders (Aho et al., 2002; van Hengel et al., 1999); thus, other possibilities must be considered as potential upstream regulators of the p120-catenin/Kaiso pathway (Figure 7).

Here, we identified Frodo as interacting with p120-catenin both in vitro and in vivo, and we have also shown Frodo to have an impact on Kaiso-mediated transcriptional repression. Frodo was initially identified in association with the DIX domain of Dsh, and, as might be anticipated given such a physical interaction, was found to modulate Wnt/β-catenin signaling (Gloy et al., 2002). We tested and found that upstream Wnt pathway components such as Dsh also have an impact on the p120-catenin/Kaiso developmental pathway. Indeed, given our previous finding that certain Wnt/β-catenin gene targets are coregulated by β-catenin/TCF and p120-catenin/Kaiso signals (Park et al., 2005), these two pathways appear to be integrated at upstream as well as downstream (promoter) levels and may act in a coupled and parallel fashion to generate more robust effects in some contexts.

In considering p120-catenin's larger roles, we cannot rule out additional signaling outputs that may or may not be independent of its effects examined here. For example, there is strong evidence that p120-catenin modulates Rho GTPase activity as well as cadherin stability (Anastasiadis and Reynolds, 2001; Davis et al., 2003), both apt to have significant downstream effects. p120-catenin has recently been implicated to regulate NF-κB signaling through Rho/ROCK (Perez-Moreno et al., 2006) and to modulate cell proliferation (Davis and Reynolds, 2006). Thus, in the future, the impact of Frodo (through p120-catenin) on Rho GTPase and other pathways will have to be considered. As an initial approach to this question, we observed that Frodo inhibits Rho activity (see Figure S4), which presumably occurs through the promotion of p120-catenin levels/function. Therefore, we are again reminded of inaccuracies that might arise when attempting to conceptually separate Wnt/β-catenin and Wnt/PCP pathway trajectories. That is, we have shown that Wnt/β-catenin signals previously established to involve the Dsh-DIX domain (and in some cases Frodo) engage the downstream actions of p120-catenin in addition to β-catenin. Even though Rho GTPases are more often viewed as mediators of Wnt/PCP pathways, our results suggest that, through p120-catenin, Rho GTPase functions may conceivably be modulated downstream of "canonical Wnt" signals as well.

How Frodo Stabilizes p120-Catenin

The means by which Frodo modulates Kaiso repressor activity may occur in more than one way. In addition to its noted interaction with Dsh, Frodo has been implicated in associating with nuclear TCF-3. It is conceivable that nuclear Frodo positively influences p120-catenin function at gene control regions (enhancing displacement of Kaiso from DNA?), especially as TCF and Kaiso themselves associate (Park et al., 2005). Another possibility is that the Frodo:p120-catenin complex enhances p120-catenin's capacity to sequester Kaiso from the nucleus in relieving Kaiso-mediated gene repression. While not exclusive of either possibility, we observe that Frodo depletion reduces p120-catenin

(H) *Xenopus* embryos were microinjected with either Myc-Dsh or HA-xp120-catenin mRNA into both cells at the two-cell stage. Injected embryos were harvested at stage 12, and either Myc-Dsh or HA-xp120-catenin was immunoprecipitated by using anti-HA or Myc antibody. Both Myc-tagged Dsh protein and HA-xp120-catenin were detected within HA-xp120-catenin and Myc-Dsh precipitates, respectively (upper blots, lanes 8 and 16). Immunoprecipitation of either HA-xp120-catenin or Myc-Dsh was successful (positive control, lanes 4 and 12). Coimmunoprecipiation of negative controls showed little or no signals (lanes 2, 6, 10, and 14). Bottom blots of whole cell/embryo extracts indicate the faithful overexpression of Myc-Dsh and HA-xp120-catenin.

(I) Direct association of p120-catenin with Frodo, but not Dsh. In vitro-translated HA-xp120-catenin, Myc-xKaiso, Myc-Frodo, and Myc-Dsh proteins were incubated at 4°C for 30 min and precipitated with anti-HA antibody. The coimmunoprecipitates were analyzed by immunoblotting with anti-HA and anti-Myc antibody. The results shown are representative of three independent experiments. The scores below selected ECL/western blots indicate the relative ratios of band intensities normalized to actin loading controls.

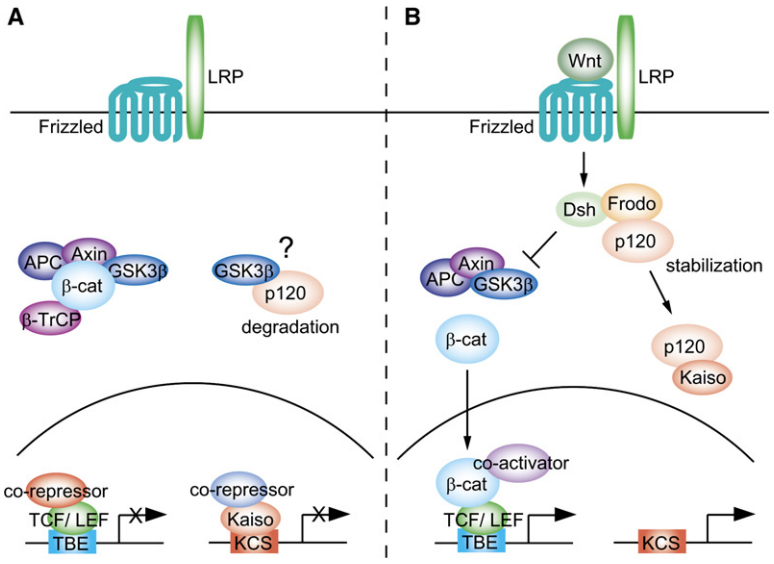

Figure 7. Model of the Frodo/p120-Catenin/Kaiso Signaling Module in Relation to Wnt Ligand Activation of Frizzled/LRP Coreceptor

(A) In the absence of Wnt ligand, the β-catenin destruction complex (APC, Axin, and GSK3β) promotes the degradation of β-catenin. p120-catenin is also subject to degradation in a GSK3β- and proteasome-modulated manner. In the reduced presence of β-catenin or p120-catenin signaling pools, TCF/LEF and Kaiso function more effectively as repressors within the nucleus.

(B) In the presence of Wnt ligand, signals are relayed from Frizzled receptor and LRP coreceptor to Dsh. Subsequently, Dsh inhibits the β-catenin destruction complex such that β-catenin is more likely to enter the nucleus to relieve repression conferred by TCF/LEF (gene activation). Simultaneously, activated Dsh interacts with Frodo through the Dsh-DIX domain and recruits and stabilizes p120-catenin. By an unknown means, 120-catenin is thought to enter the nucleus to associate with nuclear Kaiso, resulting in the derepression of Kaiso target genes, some of which are shared with TCF/LEF (not shown).

protein stability while Frodo overexpression enhances it. Moreover, Dapper, another Frd family protein, appears to positively regulate p120-catenin protein stability in the manner of Frodo, even though Frodo and Dapper may have complex roles in Wnt/β-catenin signaling (Cheyette et al., 2002; Gloy et al., 2002). When considering prior work on catenin proteins dissociated from cadherins, β-catenin has appeared more labile than p120-catenin in the absence of Wnt/β-catenin signals (Aberle et al., 1997; Logan and Nusse, 2004; Moon et al., 2004). However, we have found that lowered p120-catenin stability after Frodo depletion could be partially restored by MG132 treatment, suggesting that p120, like β-catenin, is subject to proteasomal degradation in response to Wnt signals. Other unknown mechanism may also regulate p120-catenin stability. For example, a recent report has documented Dapper-mediated lysosomal degradation of Nodal receptors and Dsh (Zhang et al., 2004, 2006).

Concerning mechanisms that might regulate p120-catenin stability, we took note of specific serine residues present within xp120-catenin's amino terminus. By comparing the amino acid sequences of p120-catenin across other animal species (analyzed by NetPhos 2.0 server, see http://www.cbs.dtu.dk/services/NetPhos/ [Blom et al., 1999]), we identified three evolutionarily conserved consensus target sequences for GSK3β. Such motifs are also present in β-catenin's amino terminus, and, when phosphorylated, they promote recognition by a large destruction complex (Axin, APC, and GSK3β) that ultimately results in β-catenin's proteasomal degradation. Although it was previously reported that p120-catenin does not associate with APC (Daniel and Reynolds, 1999), we find it intriguing that the Frodo-like Dapper has been noted to associate with GSK3β (Cheyette et al., 2002). It thus remains possible that GSK3β modulates p120-catenin stability in response to Wnt or other upstream signals, with additional mechanisms also having an impact on p120's signal tranduction capacity (Aho et al., 2002; van Hengel et al., 1999; Xia et al., 2006).

Frodo Links the Wnt/β-Catenin/TCF and p120-Catenin/Kaiso Pathways

Frodo is a positive regulator of the Wnt/β-catenin/TCF pathway at least in part via interactions with the DIX domain of Dsh (Gloy et al., 2002). Perhaps through Frodo, we additionally find that p120-catenin associates with Dsh (see Figure 6H). Since p120-catenin's levels decrease upon overexpression of Dsh-ΔDIX (an inhibitory Dsh mutant), with exogenous Frodo rescuing this effect, we believe that Wnt/β-catenin signals may modulate p120-catenin protein stability through Dsh:Frodo functional interactions. Conceivably, Frodo's serine-rich region (following its putative leucine zipper-like domain) is subject to kinase/phosphatase modulation in response to Wnt signals, determining Frodo's interaction with and stabilization of p120-catenin.

In our previous study, the p120-catenin/Kaiso and β-catenin/TCF pathways were shown to coordinately regulate certain Wnt/β-catenin target genes that harbor both Kaiso- and TCF-binding sites within their proximal promoters (Park et al., 2005). Together, our work here characterizes Frodo as a binding mate and regulator of p120-catenin, promoting p120-catenin stability and association with Kaiso, and thereby the relief of Kaiso-mediated gene repression. Thus in addition to Frodo's role as a Dsh (and TCF)-interacting protein, we point to it being a molecule that interrelates the p120-catenin/Kaiso and β-catenin/TCF signaling pathways downstream of Wnt/β-catenin stimuli. While β-catenin and p120-catenin have distinct cellular and developmental roles in a number of known contexts, our data point to novel shared functional associations of these two catenins belonging to different catenin subclasses.

Experimental Procedures

Yeast Two-Hybrid Screening

A *Xenopus* gastrula-stage cDNA expression library was constructed as indicated in the yeast pJG4-5 vector (Itoh et al., 2000). The pEG202 "bait" vector contained a fusion of the LexA DNA binding

domain and the Frodo B (middle) domain. Bait and the library vectors were transformed into the EGY48 yeast strain, and colonies were selected for growth in leucine-free medium and on X-gal-containing plates as described (Gyuris et al., 1993).

Embryo Culture, Microinjections, and In Vitro Transcription

Fertilization, embryo culture, and microinjections were performed in accordance with standard methods (Fang et al., 2004). Embryos were microinjected with capped mRNA synthesized in vitro (mMessage mMachine, Ambion) or with morpholino. All pCS2-based constructs were linearized by using NotI prior to in vitro transcription. In some experiments, the proteasomal inhibitor MG132 (Calbiochem) was present in standard 0.1× MMR incubation buffer (10 mM NaCl, 0.2 mM KCl, 0.2 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5 mM HEPES [pH 7.4]). Gastrulation phenotypes were visually scored at embryonic stages 11–12 (late gastrulation).

Antisense Oligonucleotides

xp120-catenin-morpholino (xp120-MO), Frodo-morpholino (Frodo-MO), xKaiso-morpholino (xKaiso-MO), Dapper-morpholino (Dapper-MO), and standard control-morpholino (Std-MO) were used as described previously (Cheyette et al., 2002; Fang et al., 2004; Gloy et al., 2002; Kim et al., 2004).

Constructs

Standard recombinant DNA techniques were used to construct pCS2 plasmids harboring Myc-xKaiso, HA-xKaiso, HA-xp120-catenin, and Myc-xp120-catenin. Generated previously were pCS2 plasmids harboring Myc-Dsh, Dsh-ΔDIX, Dsh-ΔPDZ, Dsh-ΔDEP, and HA-Frodo-pXT7 (Gloy et al., 2002; Park et al., 2005). Myc-Frodo-pCS2 was constructed by PCR amplification of HA-Frodo-pXT7 as a template with the following primers: Frodo-F-EcoRI, 5′-GGAATTC CAGCTCCGCCTCACCCCC-3′; Frodo-R-XbaI, 5′-GCTCTAGATCAA ACTGTTGTCATCAGCTTTAA-3′. The EcoRI/XbaI-digested PCR product was subcloned into the 6× Myc (epitope tag) pCS2 vector (pCS2-MT). Frodo deletion constructs (A–E) were generated by using endogenous BamHI, PstI, and BglII restriction enzyme sites, followed by ligation into the pCS2-MT vector.

Reverse Transcription and Semiquantitative PCR

Total RNA was extracted from *Xenopus* embryos by using Trizol (Invitrogen) according to the manufacturer's instructions. To detect *xWnt-11* and *Histone H4* transcript levels, cDNA was generated from 1 μg total RNA by using Superscript (Life Technologies, Inc.) reverse transcriptase as described by the manufacturer. PCR primer pairs were as described previously (Kim et al., 2004; Park et al., 2005).

Reporter Assays

Both wtKCS-xWnt-11 and mutKCS-xWnt-11 reporter vectors were used for luciferase assays as described (Kim et al., 2004). Briefly, reporter plasmid (0.5 ng) was coinjected with morpholino (20 ng) into the animal region of both blastomeres of two-cell embryos. Generally, three pools of eight embryos each were collected at gastrula (10.5)-stage and homogenized in 160 μl passive lysis buffer (Promega). Luciferase activities were determined from 20 μl embryo lysates by using the Luminoscan Ascent luminometer (Labsystems), and experiments were repeated three times.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation assays were performed from *Xenopus* embryo extracts as described (Park et al., 2005). Anti-Myc antibody (9E10) was used to precipitate the protein:chromatin complexes. For semiquantitative chromatin immunoprecipitation (ChIP)-PCR, primer pairs were as previously described to detect *xWnt-11*, *Siamois*, and *xLMC2* promoter fragments (Kim et al., 2004; Park et al., 2005).

Immunoprecipitation, Immunoblot, and In Vitro Translation

Monoclonal or polyclonal antibodies were employed to detect Myc-epitope (9E10), HA-epitope (12CA5), xp120-catenin (Fang et al., 2004), β-catenin (Montross et al., 2000), C-cadherin (Fang et al., 2004), p120-catenin (15D2, kindly provided by Al Reynolds), and Actin (Sigma). The anti-Frodo polyclonal antibody serum was obtained from rabbits immunized with GST-Frodo (amino acids 151–328). The serum was then depleted of anti-GST antibodies by using a GST column and anti-Frodo antibodies affinity purified by using the same GST-Frodo protein attached to AminoLink sepharose beads. For immunoprecipitation, whole-embryo lysates were prepared by using modified RIPA buffer (50 mM Tris-HCl [pH 7.4], 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, and 1 μg/ml each of Aprotinin, leupeptin, and pepstatin). All procedures were performed as described (Fang et al., 2004). For immunoblotting, procedures were performed as described. In brief, embryos were collected and lysed in 20 μl per embryo of 0.5% Triton X-100 buffer (0.5% Triton X-100, 10 mM HEPES [pH 7.4], 150 mM NaCl, 2 mM EDTA, 2 mM EGTA), inclusive of a proteinase inhibitor cocktail (Sigma). After centrifugation (14,000 rpm, 5 min), supernatant fractions were denatured with 4× SDS sample buffer (200 mM Tris-Cl [pH 6.8], 40% glycerol, 8% SDS, 200 mM DTT, 0.08% Bromophenol Blue), followed by boiling at 95°C for 5 min. A half-embryo equivalent was resolved by SDS-PAGE and transferred onto nitrocellulose membrane. For immunoblot blocking and antibody incubation, 2% bovine serum albumin-TBST (25 mM Tris-HCl [pH 8.0], 125 mM NaCl, 0.5% Tween 20) was used. Finally, SuperSignal WestPico (Pierce Biotechnology, Inc.) reagents were employed to detect HRP (horseradish peroxidase)-conjugated secondary antibodies. For in vitro translation (Promega), 1.0 μg of each mRNA (HA-xp120-catenin, Myc-xKaiso, Myc-Frodo, or Myc-Dsh) was used with 35 μl rabbit reticulocyte lysate. For protein:protein interaction tests, 10 μl of each solution was mixed for 30 min at 4°C. Precipitations employed anti-HA antibody, and coimmunoprecipitates were visualized by immunoblotting. Quantitatation of ECL/western blots was conducted by using ImageQuant software.

Mammalian Cell Culture and Immunofluorescence Microscopy

The HeLa cell line was purchased from ATCC and was maintained with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. For immunofluorescence staining, transiently transfected cells on chamber slides were fixed with 100% methanol and blocked with 2% BSA (bovine serum albumin)-PBS for 1 hr. The cells were incubated with either anti-HA or anti-Myc monoclonal antibody for 1 hr, washed with PBS, and further incubated with Texas red-conjugated secondary antibody (Santa Cruz Biotechnology, Inc). For nuclear staining, Hoechst 33258 (0.5 ng/ml, Vector Laboratories) was used. Finally, cells were observed with fluorescence microscopy (Leica, LB30S), and images were recorded by using SPOT advanced software.

Supplemental Data

Supplemental data include evidence of p120-catenin intracellular redistribution upon Frodo overexpression, lack of Frodo overexpression effects on cell-cell reaggregation, Wnt-ligand-induced reduction of Kaiso:DNA association, and reduction in the level of activated (GTP-bound) Rho GTPase after Frodo overexpression and are available at http://www.developmentalcell.com/cgi/content/full/11/5/683/DC1/.

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