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Cell Cycle Control of Wnt Receptor Activation

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SUMMARY

Low-density lipoprotein receptor related proteins 5 and 6 (LRP5/6) are transmembrane receptors that initiate Wnt/β-catenin signaling. Phosphorylation of PPPSP motifs in the LRP6 cytoplasmic domain is crucial for signal transduction. Using a kinome-wide RNAi screen, we show that PPPSP phosphorylation requires the *Drosophila* Cyclin-dependent kinase (CDK) L63. L63 and its vertebrate homolog PFTK are regulated by the membrane tethered G2/M Cyclin, Cyclin Y, which mediates binding to and phosphorylation of LRP6. As a consequence, LRP6 phosphorylation and Wnt/β-catenin signaling are under cell cycle control and peak at G2/M phase; knockdown of the mitotic regulator CDC25/string, which results in G2/M arrest, enhances Wnt signaling in a Cyclin Y-dependent manner. In *Xenopus* embryos, Cyclin Y is required in vivo for LRP6 phosphorylation, maternal Wnt signaling, and Wnt-dependent anteroposterior embryonic patterning. G2/M priming of LRP6 by a Cyclin/CDK complex introduces an unexpected new layer of regulation of Wnt signaling.

INTRODUCTION

Wnt/β-catenin signaling regulates patterning and cell proliferation throughout embryonic development and is widely implicated in human disease, notably cancer, (Clevers, 2006; Logan and Nusse, 2004; Moon et al., 2004; Polakis, 2000; Reya and Clevers, 2005; Wodarz and Nusse, 1998). Two principal classes of transmembrane (TM) receptors function to transduce Wnt/β-catenin signaling; the seven pass TM Frizzled (Fz) proteins (Bhanot et al., 1996) and the single pass TM low density lipoprotein receptor-related proteins 5 and 6 (LRP5/6; *Drosophila* Arrow; Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). Frizzled receptors activate β-catenin-dependent (canonical) as well as β-catenin-independent (noncanonical, such as planar cell polarity) pathways, while LRP5/6 function more specifically in the Wnt/β-catenin pathway (He et al., 2004).

LRP6 signaling requires Ser/Thr phosphorylation of its intracellular domain (ICD), which contains five PPPSPXS dual phosphorylation motifs comprising Pro-Pro-Pro-Ser-Pro (PPPSP) and directly adjacent casein kinase 1 (CK1) sites (see Figure S1A available online; Davidson et al., 2005; Macdonald et al., 2008; Tamai et al., 2004; Wolf et al., 2008; Zeng et al., 2005, 2008). Phosphorylation of the most N-terminal PPPSP (S1490) involves glycogen synthase kinase 3 (GSK3) (Zeng et al., 2005), while CK1g phosphorylates two Ser/Thr clusters near S1490 (see Figure S1A; Davidson et al., 2005). Phosphorylation of CK1 sites is downstream of, and requires, PPPSP phosphorylation (Davidson et al., 2005); however, alternative epistasis models have also been proposed (Yum et al., 2009). Both PPPSP and CK1 site phosphorylation is necessary for Axin binding to LRP6 and Wnt/β-catenin pathway activation (Davidson et al., 2005; Tamai et al., 2004; Zeng et al., 2005). Phosphorylated PPPSPXS motifs directly inhibit the ability of GSK3 to phosphorylate β-catenin, providing a potential mechanism linking LRP6 activation to β-catenin stabilization (Cselenyi et al., 2008; Piao et al., 2008; Wu et al., 2009). Investigating how LRP6 phosphorylation is regulated is thus crucial for understanding Wnt receptor activation and downstream signaling. We observed constitutive, non-Wnt-induced S1490 phosphorylation (Davidson et al., 2005, this study), suggesting that additional proline-directed kinases may be involved, such as the ERK or Cyclin-dependent kinase (CDK) subgroups (Manning et al., 2002).

CDKs are regulators of the cell cycle and require Cyclin partners, whose levels are precisely controlled during the cell cycle, endowing CDKs with both temporal activity and substrate specificity (Morgan, 1997). Several less well-characterized CDK-like proteins exist, including the PFTAIRE kinase subfamily (Sauer et al., 1996). Here, we report on the identification of a Cyclin/PFTAIRE-CDK complex that phosphorylates LRP6 S1490 in a cell cycle-dependent manner, which brings Wnt/β-catenin signaling under G2/M control and introduces a surprising new principle in Wnt regulation.

RESULTS

Identification of a Cyclin/CDK Required for LRP6 PPPSP Phosphorylation

One controversial question in LRP6 regulation concerns whether PPPSP phosphorylation is exclusively Wnt induced (Binnerts et al., 2007; Bryja et al., 2007; Khan et al., 2007; Zeng et al., 2005, 2008) or also constitutive (Davidson et al., 2005; Wolf

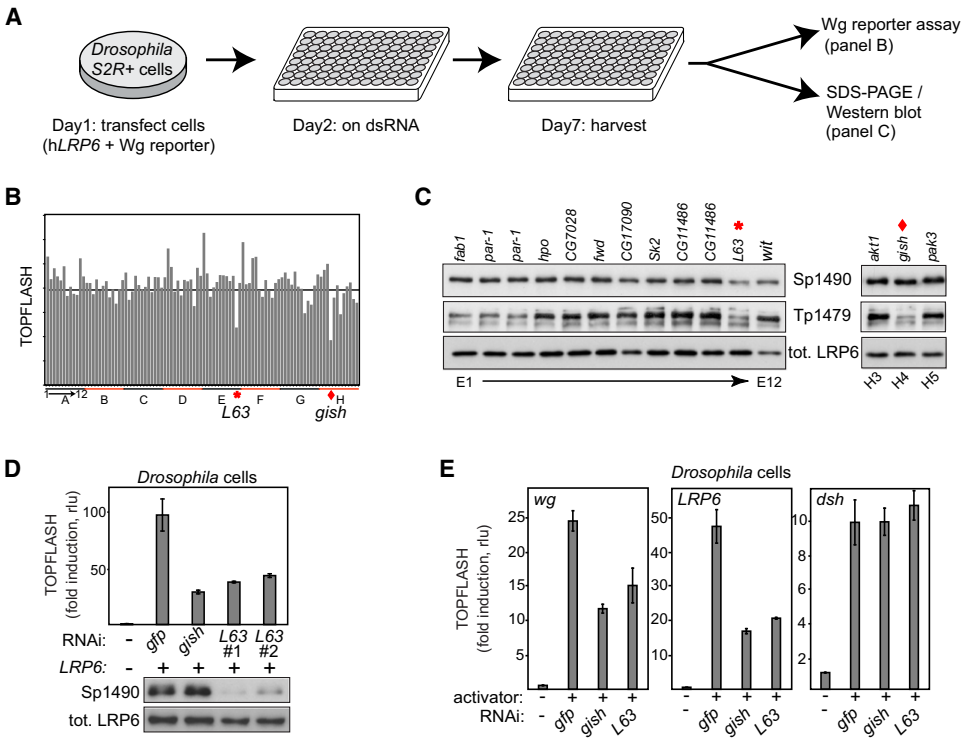

Figure 1. Identification of the PFTAIRE CDK, L63 as an LRP6 Kinase

(A) Scheme of *Drosophila* kinome-wide RNAi screen.

(B) Wg reporter assay from one of the 96-well plates screened as in (A).

(C) LRP6 western blots of lysates from row E and part of row H of the 96-well plate shown in (B), with the gene transcripts targeted by dsRNA shown above. Simultaneous reduction of LRP6 phosphorylation and Wg signaling was observed for L63 and gish RNAi (asterisks/diamonds).

(D) Wg reporter assay (upper graph) and LRP6 western blots (lower panels) from the same *LRP6* transfected *Drosophila* S2R$^+$ cells treated with indicated dsRNA (RNAi).

(E) Wg reporter assays in S2R$^+$ cells transfected with either *wg*, *LRP6*, or *dsh* as pathway activators and treated with the indicated dsRNA (RNAi).

et al., 2008). We have evidence suggesting that protein kinases in addition to GSK3 are involved in LRP6 PPPSP phosphorylation (detailed in Figure S1). To search for such additional LRP6 PPPSP kinases, we performed a kinome-wide RNAi screen, using *Drosophila* cells transfected with mammalian *LRP6* (Figure 1A). Transfection of mammalian *LRP6* without *Wingless* (*Wg*; *Drosophila Wnt*) is sufficient to induce a robust signaling response, possibly because forced receptor oligomerization upon overexpression partially bypasses Wnt requirement (Bilic et al., 2007). TOPFLASH (Wnt) reporter assay (Figure 1B) and LRP6 western blot screening (Figure 1C) expectedly identified the LRP6 kinase *gish/CK1*γ (Davidson et al., 2005), which reduced Wg signaling (Figures 1B and 1D) and T1479 phosphorylation (Figure 1C), without effecting S1490 phosphorylation levels (Figures 1C and 1D). This is in agreement with the proposed epistasis of PPPSP and CK1 site phosphorylation (Davidson et al., 2005; Zeng et al., 2005).

Importantly, we identified a single candidate PPPSP kinase, the CDK-like putative cell cycle regulator L63/Eip63E (Bettencourt-Dias et al., 2004; Stowers et al., 2000). *L63* RNAi reduced Wg signaling (Figure 1B) and phosphorylation of LRP6 at both S1490 and T1479 (Figure 1C). A second, nonoverlapping *L63* RNAi probe confirmed this effect (Figure 1D). Consistent with it acting at the level of LRP6, *L63* RNAi reduced Wg- and LRP6-induced reporter activity but had no effect on Dsh-induced signaling (Figure 1E).

Bona fide CDKs are cell cycle regulated because they depend on Cyclins for their enzymatic activity (Murray, 2004). We searched "BioGrid" database (http://www.thebiogrid.org) and found one genome-wide yeast-two-hybrid study that identified an uncharacterized *Drosophila* Cyclin, CG14939, as a potential L63 interactor (Stanyon et al., 2004). Two highly related, evolutionarily conserved homologs of CG14939 exist in most vertebrates, CCNY (Cyclin Y) and CCNYL1 (Cyclin Y-like 1). However, humans appear to have two additional members (CCNYL2 and CCNYL3). We therefore refer to *Drosophila* CG14939 as DCyclin Y. In agreement with a recent report (Jiang et al., 2009), we found that a putative N-terminal myristoylation signal localizes Cyclin Y at the plasma membrane (Figure S2A). *Dcyclin Y* RNAi phenocopied *L63* RNAi (Figure 2A), confirming it is a regulatory subunit of L63. Combining *L63* and *Dcyclin Y* RNAi only marginally enhanced these effects (Figure 2A). Like *L63* RNAi, *Dcyclin Y* RNAi reduced TOPFLASH signaling induced by Wg and LRP6, but not Dsh (not shown). We next addressed the requirement of DCyclin Y/L63 for PPPSP phosphorylation of endogenous Arrow in *Drosophila* cells. Both Sp1490 antibody and an anti-Arrow antibody recognized a *Drosophila* protein of the expected size (Figure 2B, lane 1). Confirming specific detection, the

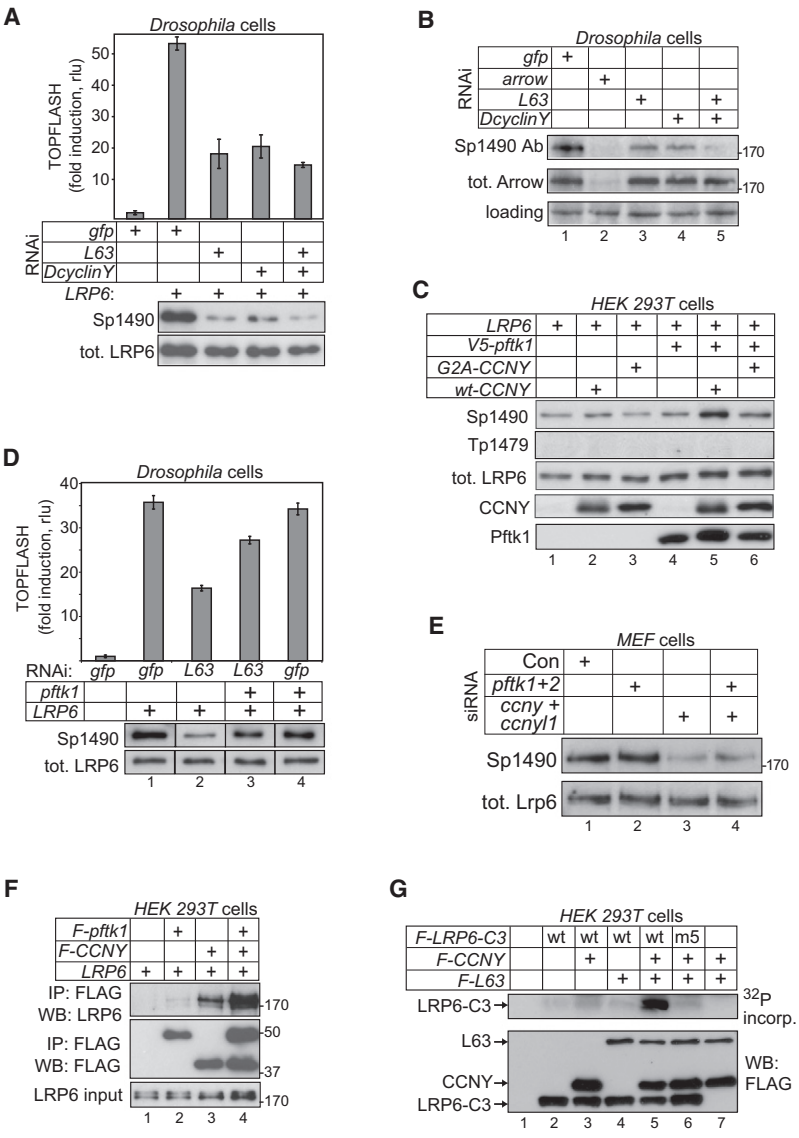

Figure 2. Cyclin Y Mediates LRP6 Phosphorylation

(A) Wg reporter assay (upper graph) and LRP6 western blots (lower panels) from the same *LRP6* transfected *Drosophila* S2R$^+$ cells treated with the indicated dsRNA (RNAi).

(B) Western blots of untransfected S2R$^+$ cells treated with the indicated dsRNA (RNAi). The loading control (lower panel) is a nonspecific band detected by Sp1490 antibody.

(C) Western blots from HEK293T cells transfected with the indicated genes.

(D) Wg reporter assay (upper graph) and LRP6 western blots (lower panels) of the same *pftk1* and/or *LRP6* transfected S2R$^+$ cells treated with the indicated dsRNA (RNAi).

(E) Western blots from MEF cells transfected with the indicated siRNAs.

(F) Western blots (WB) of immunoprecipitates (IP) or initial lysates (input) from HEK293T cells transfected as indicated.

(G) Cyclin Y(CCNY)/L63 in vitro kinase assay. Autoradiography (upper panel) and anti-FLAG WB (lower panel), shows ^{32}P incorporation and protein production controls, respectively.

Sp1490 antibody signal was phosphatase sensitive (not shown) and was eliminated by *arrow* RNAi (Figure 2B, lane 2). Using this antibody, we observed a reduced phosphorylation of Arrow with either *L63* or *Dcyclin Y* RNAi and enhanced reduction by simultaneous targeting (Figure 2B, lane 5), thus demonstrating their functional interaction. Taken together, these results support the requirement of a Cyclin/CDK complex for LRP6/Arrow PPPSP phosphorylation and Wnt signaling.

Vertebrate Cyclin Y/Pftk1 Phosphorylates LRP6

The closest vertebrate L63 homologs are the PFTAIRE CDKs, PFTK1 and PFTK2 and the related PCTAIRE CDKs, PCTK1, -2, and -3 (Charrasse et al., 1999; Okuda et al., 1992; Shu et al., 2007). We tested the ability of Cyclin Y and Pftk1 to phosphorylate PPPSP when coexpressed with LRP6. Pftk1 or Cyclin Y alone had little effect, however their combined expression with LRP6 resulted in strong enhancement (Figures 2C and S2F). The myristoylation defective Cyclin Y G2A mutant (Figure 2C), as well as Cyclin C and Cyclin I (data not shown), showed no synergy with Pftk1 in this assay. Cyclin Y/Pftk overexpression had no effect on CK1 site phosphorylation in the absence of Wnt (Figure 2C); however, they enhanced Tp1479 when cells were treated with Wnt (Figure S2B). This further confirms that S1490 phosphorylation sensitizes, or primes, downstream T1479 phosphorylation in the presence of Wnt (Davidson et al., 2005; Zeng et al., 2005). Furthermore, when expressed in *Drosophila* cells, Pftk1 rescued the decrease in S1490 phosphorylation and Wg signaling caused by *L63* RNAi (Figure 2D). Mammalian Cyclin Y overexpression likewise rescued the *Dcyclin Y* RNAi phenotype in *Drosophila* cells (Figure S2C). Knockdown of *pftk1* and *-2* by siRNA in mouse embryonic fibroblasts (MEF) and human embryonic kidney (HEK) cells was, however, without effect (Figure 2E and data not shown). This is likely due to functional compensation since all five PFTAIRE/PCTAIRE CDKs are expressed in these cells and their combined (partial) knock down was also ineffective (data not shown). In contrast, siRNAs targeting both *ccny* and *ccnyl1* mRNAs, but neither alone, reduced S1490 phosphorylation in MEF cells (Figures 2E and S2D). Furthermore, reduced LRP6 PPPSP phosphorylation in *CCNY/CCNYL1* siRNA transfected HEK cells was accompanied by reduced endogenous Wnt signaling (Figure S2E). qPCR confirmed >70% knockdown of all siRNA targeted transcripts (not shown). Consistent with these results, CCNY was very recently shown to directly interact with and regulate PFTK1 (Jiang et al., 2009).

Cyclin Y/Pftk Associate with and Phosphorylate LRP6

We next tested the directness of LRP6 phosphorylation by the Cyclin Y/CDK complex. Coimmunoprecipitation (coIP) experiments showed that LRP6 associates with Cyclin Y but not, or

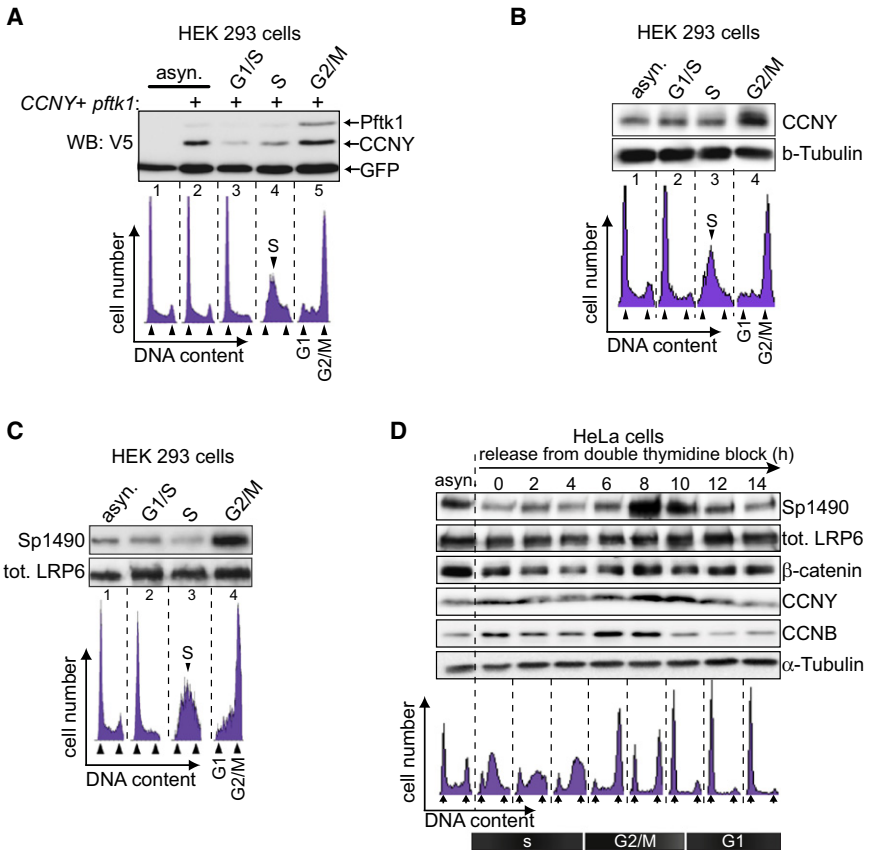

Figure 3. G2/M Cell Cycle Phase Enhancement of Cyclin Y, LRP6 Phosphorylation, and Wnt Signaling

(A–C) Western blots (WB) of V5-Pftk1, V5-CCNY, and V5-GFP (A), endogenous CCNY (B), or endogenous LRP6 (C) in asynchronous (asyn.) HEK293 cells or cells arrested at indicated phases of the cell cycle by drug treatment. FACS analysis of cells is shown beneath corresponding lanes.

(D) Western blots of endogenous proteins from HeLa cell lysates after release from double thymidine block for indicated times. FACS analysis of cells is shown beneath corresponding lanes.

(E) Normalized Wnt and BMP reporter assays of control or ligand stimulated, asynchronous, or cell cycle-arrested HEK293 cells. FACS analyses of cells are shown in Figure S3B.

(F) qPCR analysis of indicated genes in nonmitotic or mitotic HeLa cells treated for 1h with Wnt3a before mitotic shake-off. Values are normalized to GAPDH.

only weakly, with Pftk (Figure 2F). However, coexpression of Pftk with Cyclin Y enhanced coIP of LRP6 (Figure 2F) and the same was true for the *Drosophila* homologs, DCyclin Y and L63 (Figure S2F). Binding of LRP6 to the Cyclin Y/CDK complex was always accompanied by enhanced S1490 phosphorylation. Also, there was a severe reduction in the ability of G2A mutant CCNY to associate with LRP6 (Figure S2G), in agreement with its reduced activity (Figure 2C). Thus, Pftk and Cyclin Y synergize to form a functional complex with LRP6 that results in its phosphorylation. We used in vitro kinase assays to further corroborate the directness of this phosphorylation. Neither immunopurified L63 alone nor Cyclin Y alone phosphorylated a soluble, intracellular LRP6 domain (LRP6-C3; Figure 2G) but, importantly, their combination resulted in robust LRP6 phosphorylation. An LRP6 mutant (m5) that harbors Alanine point mutations within all PPPSP motifs (Tamai et al., 2004) was not phosphorylated. We conclude that Cyclin Y recruits its CDK partner to LRP6 at the membrane and phosphorylates its PPPSP motifs.

Cell Cycle Regulated Phosphorylation of LRP5/6

Since a Cyclin/CDK complex can phosphorylate LRP6 we investigated whether Wnt signaling is cell cycle regulated. Progression through the cell cycle is driven largely by regulation of CDKs, predominantly via Ubiquitin mediated degradation of their Cyclin subunits (Murray, 2004). We first demonstrated that Cyclin Y is subject to ubiquitination (Figure S3A). Pftk1 was not ubiquitinated and did not alter Cyclin Y ubiquitination; however, it was markedly enhanced/stabilized by Cyclin Y.

We then monitored overexpressed Cyclin Y and Pftk1 levels in drug synchronized HEK293 cells and observed a clear enrichment of both CCNY and Pftk1 in Nocodazole-treated cells arrested at G2/M (Figure 3A). Parallel FACS analysis confirmed cell cycle phase enrichment. We also detected clear enrichment of endogenous CCNY at G2/M (Figure 3B). Cyclin Y thus behaves like G2/M Cyclins (Murray, 2004), and this predicts that phosphorylation of LRP6 is enhanced at this stage. Indeed, endogenous Sp1490 levels showed a distinct peak in G2/M arrested cells (Figure 3C). To analyze cell cycle dependent LRP6 phosphorylation and CCNY levels with a more detailed time-course we performed double thymidine block at G1/S and release in HeLa cells. This confirmed that Sp1490 levels

peak during G2/M along with endogenous Cyclin Y, the mitotic Cyclin B, and cytoplasmic β-catenin (Figure 3D). As PPPSP phosphorylation is required for LRP6 signaling, this also predicts that Wnt signaling in HEK293 cells should be maximal at G2/M. Indeed, both low level endogenous Wnt signaling as well as Wnt3a stimulated signaling were maximal in cells arrested at G2/M with Nocodazole (Figures 3E and S3B). Lower BMP signaling at G2/M was not due to drug toxicity since BMP induced SMAD1/5/8 phosphorylation was unaffected (not shown).

To confirm that Wnt signaling is predominant at G2/M not only in pharmacologically synchronized cells but also in untreated cells we employed mitotic shake-off to enrich for G2/M cells. We used intron-specific primers to monitor nascent transcripts of the direct Wnt target gene AXIN2, which is 4-fold Wnt-inducible in HeLa cells (not shown). In the mitotic cell fraction we found a 2-fold RNA enrichment of the mitotic marker *PLK1* (Alvarez et al., 2001) and *AXIN2*, but not the control gene SMAD6 (Figure 3F). Taken together, these results indicate that Cyclin Y regulates Wnt signaling via G2/M specific phosphorylation of LRP6.

Cell Cycle-Dependent LRP6 Signalosome Formation

To further confirm the phenomenon of cell cycle-dependent Wnt signaling without using drug treatment, we performed Tp1479 immunofluorescence to detect Wnt-induced Lrp6 signalosomes (Bilic et al., 2007) in P19 cells (Figure 4). Upon Wnt treatment Tp1479 positive aggregates were detected in the majority of cells (Figure 4A); however, the signal was much stronger in mitotic cells, which are marked by their characteristic condensed chromosomes (Figures 4A and 4B). In contrast, there was no difference in BMP4 induced Smad1/5/8 phosphorylation (data not shown). siRNAs targeting *lrp5,6* significantly reduced the vesicular Tp1479 signal, confirming antibody specificity (Figure 4A). We conclude that maximal LRP6 signalosome formation occurs in mitotic cells.

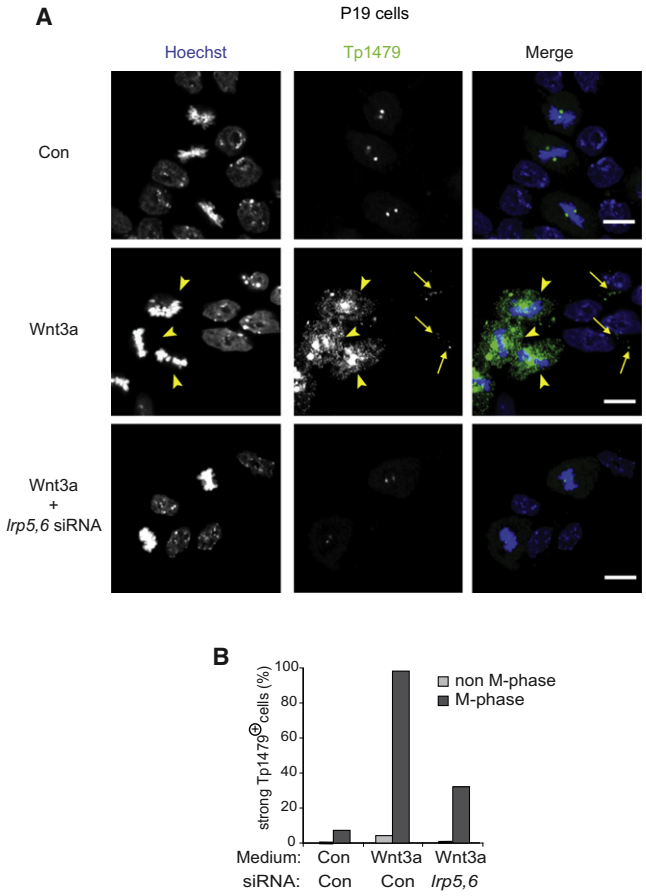

Figure 4. Phospho-LRP6 Signalosomes Predominate in Mitotic Cells

(A) Confocal microscopy of P19 cells stained with Hoechst and LRP6 Tp1479 antibody. siRNA transfected cells were treated with control or Wnt3a conditioned medium, as indicated. Arrowheads indicate M phase cells (condensed chromosomes) and arrows indicate Lrp6 signalosomes in non-M phase cells. Note that M phase cells show much stronger Tp1479 staining (vesicular clouds) compared to nonmitotic cells (few speckles). Centrosomal staining is unspecific (not significantly reduced by LRP5/6 siRNA). Bar is 10 μm.

(B) Quantification of confocal microscopy analysis shown in (A). The occurrence of strong Tp1479 positive cells was scored in non-M phase (n = 100) and M phase (n = 80) cells as distinguished by Hoechst staining.

Cyclin Y Is Required for Lrp6 Phosphorylation, Wnt Signaling, and A-P Patterning in *Xenopus* Embryos

We next addressed the physiological relevance of Lrp6 phosphorylation by the Cyclin Y/CDK complex using *Xenopus* embryos, where Wnt/β-catenin signaling regulates anteroposterior (a-p) patterning at gastrula and neurula stage (Niehrs, 2004). Both *ccny* and *ccnyl1* genes are expressed throughout early development and of the PFTAIRE and PCTAIRE CDKs *pftk2*, *pctk2*, and *pctk3* show expression at gastrula or neurula stage (Figure S4). By in situ hybridization expression of *cyclin Y* genes was not spatially restricted (data not shown).

Morpholino antisense oligonucleotides (MO) targeting *Xenopus lrp6* (Hassler et al., 2007) abolished a 200 kD band recognized by the Sp1490 antibody (Figure 5A), confirming its specific detection of endogenous Lrp6. Embryos injected with MOs targeting both *Xenopus cyclin Y* transcripts (*cycY* MO), but neither alone (not shown), showed a significant reduction in PPPSP phosphorylation (Figure 5A). The anti-Cyclin Y antibody confirmed MO knockdown. Like mammalian cell culture loss of function, injection of MOs targeting *pftks*/*pctks* had no significant effects on Lrp6 phosphorylation levels or on embryonic development (data not shown), likely reflecting functional redundancy.

Injection of *cycY* MO downregulated the Wnt reporter TOPFLASH and this was rescued by coinjection of human *CCNY* mRNA, further confirming Morpholino specificity, as well as by *β-catenin* mRNA, confirming that *cycY* MO did not unspecifically/irreversibly block Wnt signaling (Figure 5B). Downregulation was observed in early neurula but not blastula, consistent with reduction of zygotic Wnt signaling (data not shown). These results indicate an in vivo requirement for Cyclin Y in Wnt signaling.

Phenotypically, *cyclin Y* morphants were anteriorized (Figure 5C), characteristic of reduced zygotic Wnt signaling (Niehrs, 2001). Indeed, they phenocopied *lrp6* morphants (Figure 5C). Anteriorization by *lrp6* or *cycY* MO was strongest when

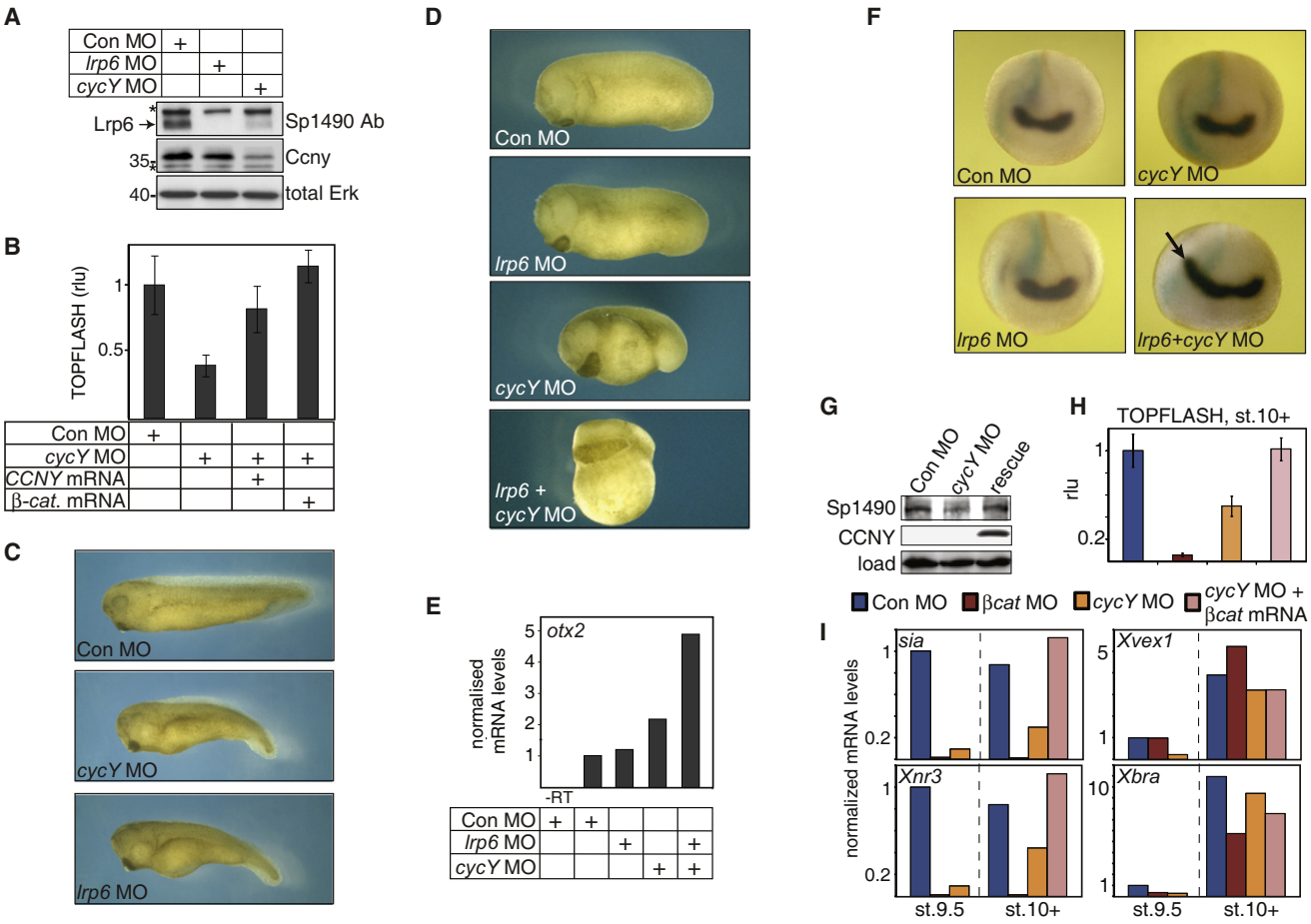

Figure 5. Cyclin Y Is Required for Wnt/β-Catenin Signaling during *Xenopus* A-P Patterning

(A) Western blots of endogenous proteins from early neurula (stage 14) *Xenopus* embryos injected equatorially into each blastomere at the four-cell stage with Morpholino oligonucleotides (MO) targeting either *lrp6* or both *cyclin Y* (*cycY* MO) transcripts. Asterisks indicate nonspecific bands.

(B) TOPFLASH reporter assays in stage 13 embryos injected as in (A) with *cycY* MOs alone or together with either 500 pg *hCCNY* mRNA or 63 pg β-*catenin-GFP* mRNA.

(C) Tailbud (stage 32) embryos microinjected with *cycY* MOs or *lrp6* MO as in (A). 0% (n = 39) of control, 61% (n = 38) of *cycY*, and 70% (n = 53) of *lrp6* MO injected embryos were anteriorized.

(D) Early tailbud (stage 28) embryos injected as in (A) with limiting MO doses. 0% (n = 30) of control, 0% (n = 42) of *cycY*, 0% (n = 63) of *lrp6*, and 91% (n = 53) of *cycY* + *lrp6* MO-injected embryos showed strong anteriorization (dorsoanterior index > 6; Kao and Elinson, 1988).

(E) qPCR analysis of *otx2* mRNA in stage 15 embryos injected as in (A) with limiting MO doses. mRNA levels were normalized to *odc*.

(F) Whole-mount in situ hybridizations for the forebrain marker *bf1* on mid-neurula (stage 15/16) embryos injected in one blastomere at the two-cell stage with either control or *cycY* MOs and/or *lrp6* MO. *Beta-galactosidase* mRNA was coinjected as tracer (blue/green staining).

(G) Western blots of endogenous proteins from mature oocytes injected equatorially with *cycY* MOs alone or together with 125 pg *hCCNY* mRNA.

(H) TOPFLASH reporter assays in *Xenopus* embryos injected equatorially as oocytes with control MO or β-*catenin* MO or *cycY* MOs. For rescue, fertilized embryos were injected with 50 pg β-*catenin-GFP* mRNA.

(I) qPCR analysis of the indicated genes in *Xenopus* embryos injected equatorially as oocytes as in (H).

equatorial, not animal, regions were injected (data not shown), indicating a predominant requirement in mesendoderm rather than ectoderm. Importantly, limiting doses of *lrp6* and *cycY* MOs synergized to anteriorize embryos (Figure 5D), consistent with a functional interaction of the proteins. Supporting this, qPCR and in situ hybridization analysis of neurula stage embryos injected with limiting doses of *lrp6* and *cycY* MO showed synergistic upregulation of the anterior neural markers, *otx2* (Figure 5E) and *bf1* (Figure 5F), respectively, a hallmark of zygotic Wnt/β-catenin inhibition.

No significant differences in the number of mitotic cells were seen in embryos injected with *lrp6* and/or *cycY* MOs, ruling out cell proliferation effects on axial patterning (data not shown). We conclude that during *Xenopus* development, Cyclin Y is required to mediate Lrp6 phosphorylation and zygotic Wnt/β-catenin signaling during a-p patterning.

We next addressed the role of maternally expressed *cyclin Y* (Figure S4) on maternal Wnt signaling (Heasman et al., 2000). *CycY* MO reduced maternal Lrp6 PPPSP phosphorylation in oocytes and this effect was rescued by *CCNY* mRNA

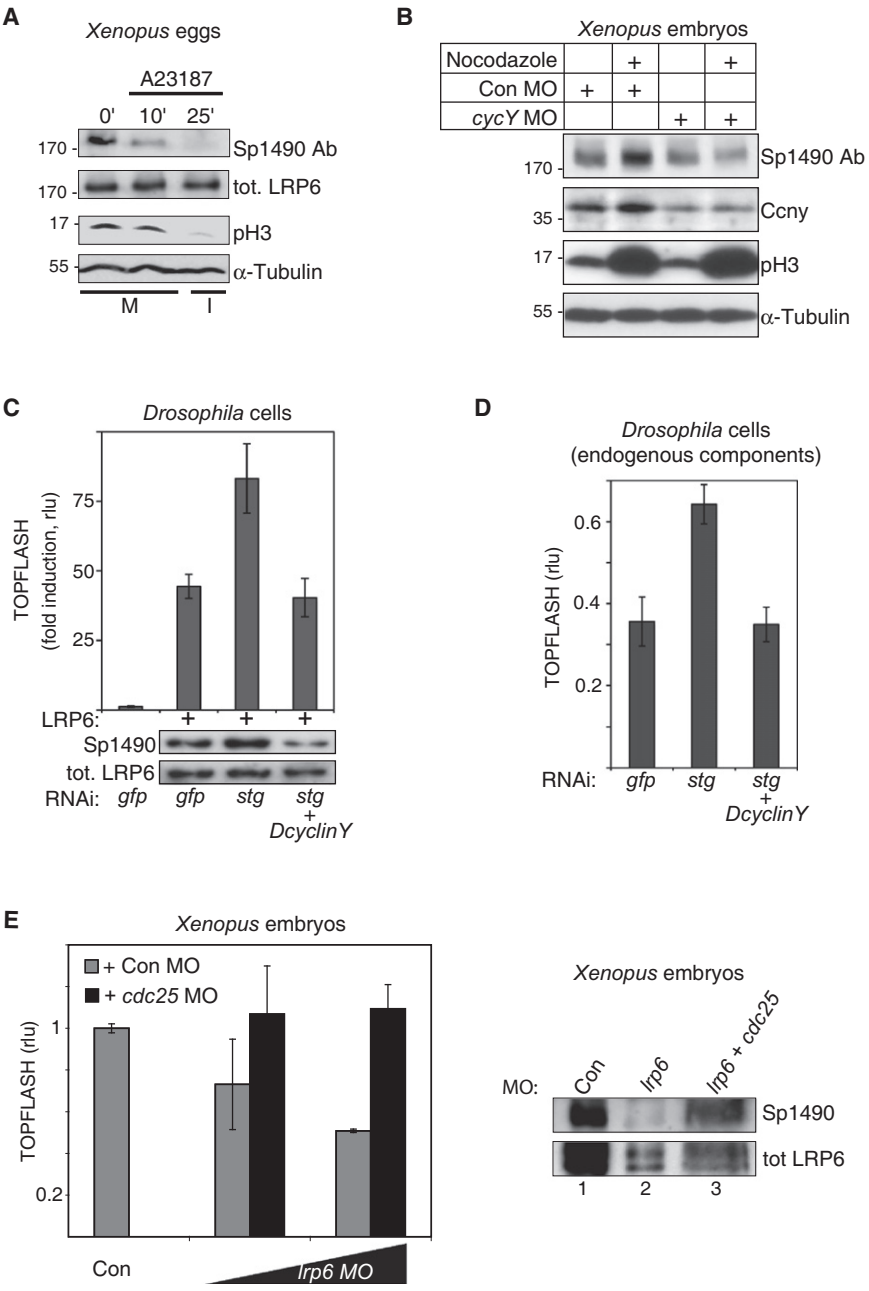

Figure 6. Cell Cycle Regulation of Lrp6 Phosphorylation and Wnt Signaling In Vivo

(A) Western blots of endogenous proteins from *Xenopus* eggs treated with the calcium ionophore A23187 for indicated times (minutes). M, metaphase II; I, interphase.

(B) Western blots of endogenous proteins from lysates of late neurula stage (stage 18) *Xenopus* embryos injected with control or *cycY* MOs as in Figure 5A, and, where indicated, treated for 8 hr with nocodazole.

(C) Wg reporter assay (upper graph) and LRP6 western blots (lower panels) from the same *LRP6* transfected *Drosophila* S2R$^+$ cells treated with the indicated dsRNA (RNAi).

(D) Wg reporter assay of S2R$^+$ cells transfected with reporter plasmids only (measuring endogenous Wnt activity) and treated with the indicated dsRNA (RNAi).

(E) Wnt reporter assays (left panel) in stage 11$^+$ *Xenopus* embryos injected equatorially at the four-cell stage with control MO or *lrp6* MOs alone or together with *cdc25B* MO. Western blots (right panel) of endogenous proteins from stage 11$^+$ *Xenopus* embryos injected with control MO, or *lrp6* MO alone or together with *cdc25B* MO. Note the strong increase of Sp1490 by cdc25 MO (compare lanes 2 and 3).

(Figure 5G). Using the host transfer technique (Mir and Heasman, 2009), we then monitored the effect of maternal Cyclin Y depletion on Wnt signaling. *CycY* MO-injected oocytes did not survive past early neurula, precluding a phenotypic analysis. However, at late blastula and early gastrula, there was a clear inhibition of both TOPFLASH reporter (Figure 5H) and expression of the direct maternal Wnt target genes *siamois* and *Xnr3* (Figure 5I). These effects were specifically due to reduced Wnt signaling, since they were rescued by β-*catenin* and since *Xvex1* and *Xbra* levels (which are regulated by BMP, FGF, and nodal) were not significantly altered (Figures 5H and 5I).

We conclude that Cyclin Y is required for Wnt signaling during early *Xenopus* development.

Cell Cycle Regulation of Lrp6 Phosphorylation and Wnt Signaling in *Xenopus*

To analyze cell cycle dependence of both PPPSP phosphorylation and Wnt signaling in *Xenopus*, we first used unfertilized *Xenopus* eggs, which are naturally arrested at metaphase II, when Lrp6 PPPSP phosphorylation is expected to be maximal. *Xenopus* eggs can be released from metaphase II arrest with the Ca^{2+} ionophore A23187 (Higa et al., 2006; Murray, 1991). Strikingly, phospho-Lrp6 levels decreased within 10 min of A23187 treatment (Figure 6A). Concomitant reduction of G2/M specific phospho-Histone H3 confirmed release from metaphase. This indicates that Lrp6 phosphorylation is cell cycle regulated maternally, in agreement with the maternal Cyclin Y depletion data.

Second, we tested whether G2/M arrest by Nocodazole induced Sp1490 in *Xenopus* embryos. Nocodazole treatment indeed upregulated endogenous Lrp6 phosphorylation. Importantly, this upregulation was blocked by *cycY* MO injection (Figure 6B), indicating that Cyclin Y confers the LRP6 responsiveness to G2/M phase. Analysis of pH3 confirmed the increase in mitotic cells in Nocodazole treated embryos.

Next, we sought to test the role of cell cycle on Wnt signaling in *Xenopus* embryos without recourse to pharmacological inhibitors. The CDC25 cell cycle regulators (CDC25A, B, and C) are phosphatases that control mitotic progression at G2/M by

activating CDK1 (Gautier et al., 1991; Strausfeld et al., 1991). Consistent with our data, it was recently reported that intestinal cells of CDC25 triple-knockout mice arrest at G2/M and have enhanced Wnt signaling (Lee et al., 2009). We therefore reproduced this result and asked if the effect is Cyclin Y dependent.

We first tested the effect of *string* (*stg*; *Drosophila CDC25*) RNAi on Wg signaling and LRP6 phosphorylation in *LRP6* transfected S2R$^+$ cells. Consistent with G/2M arrest, *stg* RNAi resulted in fewer and larger cells (Edgar and O'Farrell, 1989; not shown). As predicted, it also increased both Wg signaling and LRP6 phosphorylation. Importantly, *Dcyclin Y* RNAi blocked these effects (Figure 6C), confirming that Cyclin Y confers the LRP6 responsiveness to G2/M phase. *Stg* RNAi also enhanced TOPFLASH activity from endogenous *wg* signaling in a Cyclin Y-dependent manner (Figure 6D). We next depleted CDC25B in *Xenopus* embryos using a published Morpholino (Ueno et al., 2008) to ask if this would increase Wnt signaling or Lrp6 phosphorylation in gastrulae, similar to *Drosophila* cells. By itself, *cdc25B* MO had no significant effect on TOPFLASH reporter in wild-type gastrulae, possibly because phospho-LRP6 is not limiting. We therefore sensitized the system by Morpholino-depletion of LRP6, which reduced total LRP6, phospho-LRP6 and TOPFLASH activity (Figure 6E). Importantly, injection of *cdc25B* MO increased both TOPFLASH activity and phospho-LRP6, but not total LRP6. This neutralization of one Morpholino effect by another is a striking confirmation of the G2/M input into LRP6 regulation.

Taken together, these results provide compelling evidence that LRP6 phosphorylation at G2/M by a Cyclin Y/CDK complex promotes Wnt/Wg signaling not only in *Drosophila*- and mammalian-cultured cells but also in a developing embryo.

DISCUSSION

An important issue in the field of Wnt/β-catenin signaling concerns the regulation of LRP5/6/Arrow function via phosphorylation. Here, we have identified the unusual plasma membrane tethered Cyclin Y/PFTAIRE complex which functions predominantly at the G2/M phase of the cell cycle to phosphorylate the PPPSP motifs of LRP6. The results suggest a G2/M priming model of LRP5/6/Arrow phosphorylation, where the Cyclin Y/CDK complex phosphorylates LRP6 at PPPSP motifs, which then primes adjacent phosphorylation by CK1 (Figure 7A). However, PPPSP priming alone is not sufficient for phosphorylation by CK1, as Wnt-induced LRP6 aggregation is also required (Bilic et al., 2007). Combined phosphorylation at PPPSP and CK1 sites then promotes Gsk3-Axin binding to LRP6 and signalosome formation. Since GSK3 and Cyclin Y/CDK are both essential for LRP6 priming they apparently act nonredundantly. So why is there a dual kinase input to PPPSP phosphorylation? The phosphorylation of LRP6 by GSK3 occurs in acute response to Wnt signaling and it was suggested that it serves to amplify receptor activation (Macdonald et al., 2008; Wolf et al., 2008; Zeng et al., 2005, 2008). Cyclin Y/CDK phosphorylates Wnt independently at G2/M, thereby gating signal transduction in proliferating cells. One possibility is that individually both kinases prime LRP6 substoichiometrically at the five PPPSP sites and that only their combined action is sufficient for full LRP6 signaling competence.

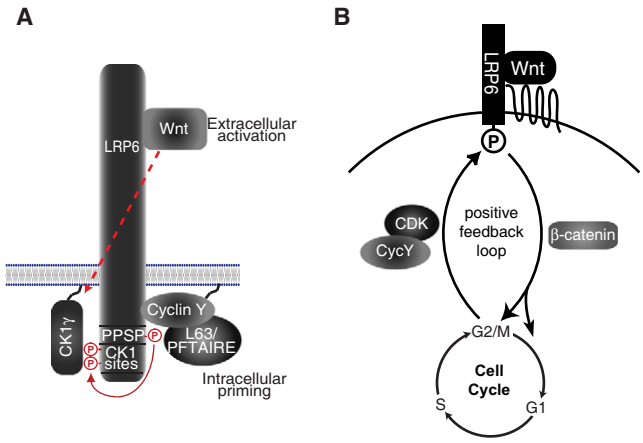

Figure 7. G2/M Priming Model for Cell Cycle Control of Wnt Receptor Activation

(A) G2/M priming model of LRP6 phosphorylation. PPPSP phosphorylation by Cyclin Y/CDK primes LRP6 for subsequent CK1γ mediated phosphorylation upon extracellular Wnt stimulation. Combined phosphorylation at PPPSP and CK1 sites promotes Axin binding (not shown for simplicity). Dashed arrow represents Wnt-dependent phosphorylation of LRP6 at CK1 sites by CK1γ. Wnt induced, GSK3-mediated PPPSP phosphorylation is not shown for simplicity.

(B) Positive feedback loop between cell cycle and Wnt receptor activation. Note that Wnt/LRP6 signaling impacts the cell cycle both by promoting G1 progression as well as by directly regulating the mitotic apparatus.

Cell Cycle Regulation of Wnt Signaling

Our findings have important implications for the link between proliferation and Wnt signaling. It has been long known that there is cross talk between mitogenic growth factors and Wnt signaling (Shackleford et al., 1993; Dailey et al., 2005; Fodde and Brabletz, 2007; Katoh and Katoh, 2006; Ten Berge et al., 2008). Our results may explain why mitogenic growth factors synergize with Wnt/β-catenin signaling, namely by G2/M priming of LRP6 through enhanced cell proliferation, which sensitizes LRP6 for incoming Wnt signals. Moreover, not only extracellular but also intracellular cell cycle check point regulators controlling G2/M entry are likely to affect Wnt signaling.

Wnt/β-catenin signaling itself promotes G1 progression by inducing *c-myc* and *cyclin D1* (He et al., 1998; Tetsu and McCormick, 1999). This suggests that Wnt/β-catenin signaling can entrain a positive feedback loop in proliferating cells by promoting cell cycle progression, which triggers LRP6 phosphorylation at G2/M (Figure 7B). Simultaneous stimulation by Wnt and mitogenic growth factors could initiate such a loop. Indeed, our results may explain the previously noted G2/M enrichment of β-catenin and Wnt signaling (Olmeda et al., 2003; Orford et al., 1999). Likewise, protein levels of the direct Wnt target gene *Axin2*, considered a marker gene for Wnt/β-catenin signaling, also peak during mitosis (Hadjihannas et al., 2006).

What may be the function of a Wnt positive feedback loop during the cell cycle? One of the many roles of Wnt/beta-catenin signaling is to promote cell proliferation and the positive feedback loop suggested by our study may enhance the systems' levels properties of the cell cycle. Specifically, the loop may promote synchrony of cell cycle regulated events or constitute a bistable switch between cell proliferation and cell cycle exit.

One interesting question raised by our study concerns preferential transcription of Wnt target genes around G2/M. Most genes are transcriptionally silenced between late prophase and early telophase (Gottesfeld and Forbes, 1997), yet TOPFLASH reporter and AXIN2 peak around G2/M. It will therefore be interesting to investigate whether Wnt target genes are transcribed during the more permissive stages G2, early prophase, or late telophase.

Another important question raised by our study is whether G2/M priming is essential or only modulatory for Wnt/β-catenin signaling in general, in particular in light of Wnt signaling in nondividing cells. The fact that LRP6 signaling is promoted by G2/M phase does not exclude Wnt/β-catenin signaling in other cell cycle phases or in nondividing cells. Even though during interphase the levels of LRP6 signalosomes, Sp1490, β-catenin, and reporter activation are lower compared to G2/M, such Wnt/β-catenin signaling is likely physiologically relevant and may involve additional PPPSP kinases, such as GSK3. Surprisingly little is known about Wnt/β-catenin signaling in nondividing cells. In transgenic Wnt-reporter mice, Wnt activity is detected in apparently postmitotic cells in the adult brain, retina, and certain liver cells (Liu et al., 2007). In the adult liver, Wnt/β-catenin signaling controls perivenous gene expression (Benhamouche et al., 2006). Furthermore, Wnts play a role in axon remodeling in postmitotic neurons (Salinas, 2005) and at least one study suggests that this can involve the β-catenin pathway (Zaghetto et al., 2007). In light of our results it will be interesting to examine more systematically Wnt/β-catenin signaling and in particular the LRP6 kinases involved in postmitotic cells.

Wnt/β-Catenin Signaling and Mitosis

Traditionally it is thought that Wnt/β-catenin signaling acts to regulate gene expression of downstream targets (Logan and Nusse, 2004; Stadeli et al., 2006). Why then should Wnt/β-catenin signaling peak at G2/M? One likely answer is that components of the Wnt/β-catenin pathway play a crucial role during mitosis beyond transcriptional activation. In *C. elegans*, Wnt signaling regulates the orientation of the mitotic spindle in early development (reviewed in Walston and Hardin, 2006). In mammalian cells, phosphorylated β-catenin itself binds to centrosomes and is involved in spindle separation during mitosis (Bahmanyar et al., 2008; Huang et al., 2007; Kaplan et al., 2004). Likewise, GSK3, Adenomatous polyposis coli protein (APC) and Axin2, which are components of the β-catenin destruction complex, also have direct functions in mitosis (Kockeritz et al., 2006; Aoki and Taketo, 2007; Hadjihannas et al., 2006). Taken together these data suggest that Cyclin Y/CDK phosphorylates LRP6 at G2/M to induce Wnt/β-catenin signaling for orchestrating a mitotic program.

EXPERIMENTAL PROCEDURES

Antibodies

Rabbit polyclonal anti-Cyclin Y1 antibody was raised against a synthetic peptide (NH2-CPRWSPAIIS-COOH) and affinity-purified. Rabbit polyclonal Sp1490, Tp1479, and T1479 antibodies were as described (Davidson et al., 2005). Anti-Arrow antibody was a kind gift from S. DiNardo. Other antibodies used were: anti-FLAG, anti-Erk, alpha-Tubulin, anti-β-Catenin (Sigma); anti-pSmad1/5/8, anti-LRP6 (C5C7); anti-GSK3α (Cell Signaling); anti-β-Actin, anti-phospho-Histone 3-Ser10 (pH 3) (Abcam); anti-CCNB (Transduction Laboratories).

Kinome-wide RNAi Screen for LRP6 PPPSP Kinases

Ten-centimeter plates of *Drosophila* S2R$^+$ cells were transfected with 2.5 μg of *pAc-LRP6*, 1.5 μg *LEF7-luc*, 1.5 μg *pPacPL-lef1*, 500 ng *pAc-EYFP*, and 5 μg empty *pAc5.1* vector and 16 hr later cells were added to 96-well plates prearrayed with dsRNAs, targeting all annotated *Drosophila* kinases, for gene silencing as described (Boutros et al., 2004). After 5 days, cells were harvested in 50 μl passive lysis buffer (plb, Promega), immediately split in two and 6 μl of 5× cholate buffer (5% sodium cholate, 200 mM Tris-HCl [pH 7.0], 750 mM NaCl, 50 mM NaF, 25 mM Na_3VO_4, 5 mM PMSF, and protease inhibitors) was added to one-half which was vortexed, spun, and 20 μl clarified supernatant added to 7 μl 4× SDS-PAGE loading buffer. SDS-PAGE/western blot analysis was performed with Sp1490, Tp1479, and T1479 antibodies to detect LRP6 PPPSP and CK1 cluster 1 phosphorylation levels and total LRP6 levels, respectively. Remaining lysates in plb were used for Wg luciferase reporter assays.

Plasmids

L63 open reading frame was subcloned into pCS2$^+$, as were human *CYCLIN Y*, *Drosophila cyclin Y (CG14939)*, and mouse *pftk1*. Where indicated, N-terminal FLAG or V5 and C-terminal EGFP tags were added. The G2A CCNY mutant clones were generated by PCR. In Figure 2D mouse *pftk1* subcloned into *pActin5.1* was used. Other constructs were previously described (Davidson et al., 2005; Miller and Moon, 1997).

Cell Culture, Luciferase Reporter Assays, coIP Assays, and siRNA Transfection

Drosophila S2R$^+$ cells were maintained at 25° C in Schneider's medium containing 10% FCS. HEK293T, HEK293, and mouse embryonic fibroblast (MEF) cell lines were maintained at 37° C and 10% CO_2 in DMEM containing 10% FCS. Mouse Wnt3a conditioned medium was produced from mouse L cells stably transfected with mouse *wnt3a* and control conditioned medium was from nontransfected L cells (ATCC CRL-2647 and CRL-2648, respectively; Shibamoto et al., 1998).

For S2R$^+$ Wg reporter assays and/or WB the following amounts of DNA were transfected in 6-well plates using Effectene (QIAGEN): 500 ng of *pAc-human-LRP6*, *pPacPL-wg* and *Mt-dsh*; 250 ng of *pAc5.1-mouse-pftk1*, *pAc-human-CCNY*, *LEF7-luc* (TOPFLASH), and *pPacPL-lef1*; 125ng *pRp128-Rluc* (Renilla control). Sixteen hours later, cells were added to dsRNA for gene silencing as described (Bartscherer et al., 2006). *Dsh* expression was induced with 500 μM $CuSO_4$ 24 hr before cell harvest. dsRNAs were checked for off target effects using E-RNAi (Arziman et al., 2005; http://rnai.dkfz.de).

For Figure 2C, HEK293T cells were transfected in 24-well plates using jetPEI transfection reagent. Amounts transfected were: *pCS-human-LPR6*, 100 ng; *pCMV-mouse-mesd,* 25 ng; *pCS-V5-mouse-pftk1,* 400 ng; *pCS-human-CCNY-wild-type or G2A* mutant, 12.5 ng. Twenty-four hours after transfection, cells were harvested in 1% Triton lysis buffer (1% Triton X-100, 50 mM Tris-HCl [pH 7.0], 150 mM NaCl, 25 mM NaF, 5 mM Na_3VO_4, 5 mM EDTA and protease inhibitors) for western blot analysis.

For luciferase reporter assays in Figure 3E, HEK293 cells in 6-well plates were transfected in triplicate with 160 ng TOPFLASH reporter plasmid using Fugene 6, synchronized as described under "Cell Cycle and Flow Cytometry" and stimulated for 8 hr with control or Wnt3a conditioned medium or 50 ng/ml of control (BSA) or recombinant BMP4 protein (R&D). During Wnt and BMP treatment the drug concentrations were reduced to half to maintain cell cycle arrest at G1/S and G2/M. Cells were collected and either fixed for flow cytometry or lysed for luciferase assay. Luciferase activity was normalized to total protein content by BCA method (SIGMA). For BMP reporter assay, a stable HEK293 cell line harboring a BREx4 element (Hata et al., 2000) in a dLuc reporter (Ueda et al., 2005) was used. For siRNAs, Dharmacon Smartpools were transfected using Dharmafect 1 (Dharmacon) according to the manufacturer.

All error bars shown are SD from mean of triplicates.

Embryos, Oocytes, In Situ Hybridization, Luciferase Reporter Assays, and RT-PCR

In vitro fertilization, embryo culture, staging, preparation of mRNA, microinjection, and in situ hybridization were carried out as described (Gawantka

et al., 1995). Whole-mount *Xenopus* immunostaining was carried out using anti-pH3 essentially as described (Sive et al., 2000).

Stage 6 oocytes were manually defolliculated, injected equatorially with MOs or mRNA, and cultured a total of 60–72 hr at 18° C in oocyte culture medium (OCM). Oocyte maturation was stimulated with 2 μM progesterone in OCM for 12 hr (Zuck et al., 1998). For western blot shown in Figure 5G, MOs were injected into oocytes just after defolliculation, and mRNA was injected 48 hr after. Oocytes were harvested after maturation and processed for WB. For qPCR analysis shown in Figure 5I, oocytes were injected with MOs after defolliculation and fertilized using the host transfer technique (Mir and Heasman, 2009). Embryos were sorted from host embryos and maintained in 0.3× Barth. At four-cell stage, embryos were injected with reporters together with either control mRNA or *β-catenin-GFP* mRNA.

For *Xenopus*, Wnt reporter assays shown in Figures 5B, 5H, and 6E, 50 pg *TOPFLASH* DNA containing three copies of the TCF-binding site, together with 12.5 pg *pRLTK* (Renilla) DNA for normalization, was injected equatorially into four-cell stage *Xenopus* embryos together with MOs and mRNAs. Luciferase assays were carried out using the Promega dual luciferase assay system.

qPCR assays were performed using an LC480 light cycler (Roche). PCR primers used for amplification of *Xenopus laevis* transcripts are provided in Table S1. Morpholino antisense oligonucleotides (Gene Tools) were designed to target both *Xenopus laevis* and *tropicalis ccny* and *ccnyl1* and have the following sequences: *ccny*, 5′-CACAGCAGGATGTGGTGTTCCCCAT-3′; *ccnyl1*, 5′-CGCAACAGGTCACGGTGTTCCCCAT-3′. *Lrp6* (Hassler et al., 2007) and *cdc25B* Morpholinos (Ueno et al., 2008) have been described previously. Equal amount of MO were injected by adjustment with the standard control MO, where necessary. Doses injected (ng/embryo) were 2–6 (*lrp6*), 40–80 (*ccny* + *ccnyl1* mix, 1:1), 80 (*cdc25*); ng/oocyte: 13–30 (*ccny* + *ccnyl1* mix, 1:1), 20 (*β-catenin*).

For western blot analysis, whole *Xenopus* embryos or oocytes were homogenized in Triton lysis buffer at 1 embryo/oocyte per 10 μl, cleared twice by centrifugation (20,000 × g, 5 min at 4°C), heated at 99°C for 2 min with SDS loading buffer, and analyzed by SDS-PAGE.

Egg extracts for Figure 6A were prepared essentially as described (Murray, 1991) from eggs treated or not treated with 5 μM A23187 in presence of 100 μg/ml cycloheximide (Sigma) and homogenized in Triton lysis buffer.

In Vitro Kinase Assays

FLAG-LRP6C3 (10 μg), *FLAG-C3m5* mutant (5 μg), *FLAG-L63* (6 μg), *FLAG-CCNY* (3 μg), and pCS2$^+$ (5 μg) were transfected separately in HEK293T cells in 10 cm dishes. After 40 hr, cells were lysed in buffer containing 2% NP40; lysates from *LRP6-C3*, *-C3m5*, or control transfected cells were immunoprecipitated on FLAG-M2 beads for 1 hr at 4°C, then lysates from *L63* and/or *CCNY* or control transfected cells were coimmunoprecipitated on the beads for 1 hr at 4°C as indicated in Figure 2G. Beads were washed once with 2% NP40 buffer containing 0.5 M NaCl and equilibrated in kinase buffer lacking ATP. Reactions were initiated by combining 50 μl beads with 50 μl of kinase buffer supplemented with 10 μCi ATP and incubated for 1 hr at 30°C; kinase reaction buffer contained 80 mM Na-β-glycerolphosphate, 15 mM $MgCl_2$, 20 mM EGTA, 0.1 mM Na_3VO_4, 1.0 mM NaF, 1 mM DTT, 50 μM ATP. Reaction products were washed once with cold PBS, dissolved in 50 μl SDS loading buffer (95°C, 5 min) and supernatants analyzed by autoradiography and western blot.

Cell Cycle and Flow Cytometry

For G1/S arrest, HEK293 cells were treated with 4 μg/ml Aphidicolin for 16 to 24 hr. For S-phase cells, Aphidicolin-arrested cells were washed with PBS and released into growth for 8 hr. For G2/M arrest, cells were treated with 100 ng/ml Nocodazole for 16 to 24 hr. Cell cycle states were confirmed by flow cytometry after trypsinization, fixation in 70% ethanol, and staining with 50 μg/ml propidium iodide for 30 min at 37° C. Cells (at least 10,000) were analyzed on a FACSCalibur (BD). For western blot analysis after synchronization, HEK293 cells were harvested either directly in Triton buffer for total cell lysates (Figures 3A and 3B) or, for membrane fractions (Figure 3C), homogenized in hypotonic buffer (5 mM HEPES [pH 7.0], 1 mM $MgCl_2$, 10 mM Na-Pyrophosphate, 10 mM NaF, 5 mM Na_3VO_4, and protease inhibitors) with 40 Dounce strokes, nuclei removed by low-speed centrifugation (2500 rpm, 5 min, 4° C) then membranes pelleted (20,000 × g, 10 min, 4°C), dissolved in SDS loading buffer, heated at 95° C for 5 min and analyzed by SDS-PAGE.

For double thymidine block in Figure 3D, HeLa cells were treated 2 × 19 hr with 2 mM thymidine, with 9 hr recovery between treatments. G1/S arrested cells were then washed to progress through the cell cycle. Cells were fixed for FACS analysis or fractionated for western blot analysis as described above. SDS-PAGE/WB was performed on both cytosolic (β-catenin, CCNB, Tubulin) and membrane fractions (LRP6, CCNY).

For mitotic shake-off, HeLa cells were treated with control or Wnt3a conditioned medium for 1 hr and culture dishes gently tapped. Dislodged (mitotic) and adherent (nonmitotic) cells were pelleted by centrifugation then split for either RNA extraction using TRIZOL reagent or FACS analysis after ethanol fixation. qPCR primers used are shown in Table S1.

Immunofluorescence Analysis

For P19 immunofluorescence staining, cells were reverse transfected in 6-well plates with 100 nM control or *LRP5/6* siRNAs (Dharmacon) using Lipofectamine 2000 (Invitrogen). After 1 day, cells were re-plated on glass coverslips in 10 cm dishes. 24 hr after replating, cells were treated with control or Wnt3a-conditioned medium containing 3 μM Epoxomicin (SIGMA) for 3 hr and immunostained as described (Bilic et al., 2007). Endogenous phosphorylated LRP6 signalosomes were detected with anti-Tp1479 followed by goat anti-rabbit Alexa-488 (Molecular Probes). The Tp1479 antibody was cleared after overnight incubating at 4° C with *Xenopus* egg extract prebound to activated CH-Sepharose 4B (SIGMA). Nuclei were visualized by Hoechst staining. Fixed samples were examined on a confocal laser scanning microscope (Nikon C1si).

SUPPLEMENTAL DATA

Supplemental Data include four figures and one table and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00480-8.

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