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; Rey 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; Wehli 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). 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 et al., 2008). A recent report shows that the proline-directed kinase PFTK is required for LRP6 S1490 phosphorylation under Wnt-independent conditions, suggesting that PPPSP phosphorylation may be a constitutive process (Davidson et al., 2005). However, the mechanism by which LRP6 phosphorylation is regulated when Wnt is not activated is not well understood. To address this question, we performed a kinome-wide RNAi screen to identify factors required for LRP6 PPPSP phosphorylation.

Using a lentiviral shRNA library targeting 8,800 human kinases, we identified several factors that specifically impaired LRP6 PPPSP phosphorylation in a Wnt-dependent manner. Among these, the Drosophila Cyclin-dependent kinase L63, which is highly conserved in vertebrates, was found to be essential for LRP6 PPPSP phosphorylation. Knockdown of L63 using specific shRNAs significantly reduced PPPSP phosphorylation of LRP6 in HEK293 cells (Figure 1A). To confirm the role of L63 in PPPSP phosphorylation, we co-expressed LRP6 with wild-type or kinase-dead (KD) L63 in HEK293 cells. Wild-type L63 rescued PPPSP phosphorylation of LRP6, while the KD version had no effect (Figure 1B). These results indicate that L63 is required for PPPSP phosphorylation of LRP6 in a Wnt-dependent manner.

Next, we examined the effect of L63 knockdown on Wnt/β-catenin signaling. To do this, we transfected HEK293 cells with shRNAs targeting L63 or control shRNA and treated them with Wnt3a. Knockdown of L63 resulted in a significant reduction of β-catenin stabilization in response to Wnt3a (Figure 1C). These results suggest that L63 is required for Wnt/β-catenin signaling.

To further support the role of L63 in Wnt/β-catenin signaling, we used a reporter assay. HEK293 cells were cotransfected with a reporter plasmid containing a β-catenin-dependent luciferase gene and wild-type or KD L63 expression vectors. Treatment with Wnt3a resulted in a significant increase in luciferase activity in cells expressing wild-type L63, but not in cells expressing KD L63 (Figure 1D). These results further support the role of L63 in Wnt/β-catenin signaling.

In summary, our results indicate that L63 is a key regulator of LRP6 PPPSP phosphorylation, which is crucial for Wnt/β-catenin signaling. These findings suggest that L63 plays a central role in the regulation of LRP6 phosphorylation and downstream signaling, providing a potential mechanism link-
Developmental Cell

Cell Cycle Regulation of LRP6 Phosphorylation

![Diagram](http://www.cell.com/DevelopmentalCell)

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

(A) Scheme of Drosophila kinase-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.
(D) Wg reporter assay (upper graph) and LRP6 western blots (lower panels) from the same Simultaneous reduction of LRP6 phosphorylation and Wg signaling was observed for L63 and gish RNAi (asterisks/diamonds).
(E) Wg reporter assays in S2R+ cells transfected with either RNAi.

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/CK1γ (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 (Betten-court-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 photoprecipitated 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
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 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., 2000). 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 phosphorylation when cells were treated with Wnt (Figure S2B). This further confirms that S1490 phosphorylation sensitzes, 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 *Drosophila* Y RNAi phenotype in *Drosophila* cells (Figure S2C). Knockdown of *pftk1* and -2 by siRNA in mouse embryonic fibroblasts (MEF) and human embryonic kidney cells (HEK) 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) knockdown was also ineffective (data not shown). In contrast, siRNAs targeting both *ccny* and *ccny1* 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
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 cells treated with control or Wnt3a conditioned medium, as indicated. Arrowheads indicate M phase cells in 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.

**Cyclin Y Is Required for Lrp6 Phosphorylation, Wnt Signaling, and A-P Pattern 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 ccny1 genes are expressed throughout early development and of the PFTAIRE and PCTAIRE CDKs ptk2, pckt1, and pckt3 show expression at gastrula or neurula stage (Figure S4). By in situ hybridization expression of cyclin Y genes was not spatially (data not shown).

Morpholino antisense oligonucleotides (MO) targeting Xenopus lrp6 (Hassler et al., 2007) abolished a 200 kDa 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 (ccyY 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 ptk2/pckt3 had no significant effects on Lrp6 phosphorylation levels or on embryonic development (data not shown), likely reflecting functional redundancy.

Injection of ccY 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 ccyY MO did not specifically/reversibly 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 ccY MO was strongest when
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 btf1 (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.
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 Xve1 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.

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. This indicates that Lrp6 phosphorylation is cell cycle regulated maternally, in agreement with the maternal Cyclin Y depletion data.

Cell Cycle Regulation of Lrp6 Phosphorylation and Wnt Signaling In Vivo

To analyze cell cycle dependence of both PPPSP phosphorylation and Wnt signaling in Xenopus, we first used unfertilized Xenopus eggs, which are normally arrested at metaphase II, when Lrp6 PPPSP phosphorylation is expected to be maximal. Xenopus eggs can be released from metaphase II arrest with the Ca2+ 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

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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).

Phosphorylation and Wnt Signaling In Vivo

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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 S2 cells. Consistent with G2/M 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/b-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 signalingosome 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.

**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 Brabietz, 2007; Katoh and Katoh, 2006; Ten Berge et al., 2008). Our results may explain why mitogenic growth factors synergize with Wnt/b-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/b-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/b-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 b-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/b-catenin signaling, also peak during mitosis (Hadjiyanass 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/b-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/b-catenin signaling in other cell cycle phases or in nondividing cells. Even though during interphase the levels of LRP6 signalosomes, Sp1490, b-catenin, and reporter activation are lower compared to G2/M, such Wnt/b-catenin signaling is likely physiologically relevant and may involve additional PPPSP kinases, such as GSK3. Surprisingly little is known about Wnt/b-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/b-catenin signaling controls periventricular 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 b-catenin pathway (Zaghetto et al., 2007). In light of our results it will be interesting to examine more systematically Wnt/b-catenin signaling and in particular the LRP6 kinases involved in postmitotic cells.

**Wnt/b-Catenin Signaling and Mitosis**

Traditionally it is thought that Wnt/b-catenin signaling acts to regulate gene expression of downstream targets (Logan and Nusse, 2004; Stadler et al., 2006). Why then should Wnt/b-catenin signaling peak at G2/M? One likely answer is that components of the Wnt/b-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 b-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 b-catenin destruction complex, also have direct functions in mitosis (Koerner et al., 2006; Aoki and Taketo, 2007; Hadjihannas et al., 2006). Taken together these data suggest that Cyclin Y/CDK phosphorlates LRP6 at G2/M to induce Wnt/b-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-7-Catenin (Sigma); anti-pSmad1/5/8, anti-LRP6 (CSC7); anti-GSK3a (Cell Signaling); anti-p-Actin, anti-phospho-Histone 3-Ser10 (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 mg of pAc-LRP6, 1.5 mg LEFT-7-luc, 1.5 mg pPACP-lrif1, 500 ng pAC-EYFP, and 5 mg empty pAc5.1 vector and 16 hr later cells were added to 96-well plates prearayed with dsRNAs, targeting all annotated Drosophila kinases, for gene silencing as described (Boutros et al., 2004). After 5 days, cells were harvested in 50 ml passive lysis buffer (pbl, Promega), immediately split in two and 6 ml of 5x cholate buffer (5% sodium cholate, 200 mM Tris-Cl [pH 7.0], 750 mM NaCl, 50 mM NaF, 25 mM Na2VO4, 5 mM PMSF, and protease inhibitors) was added to one-half which was vortexed, spun, and 20 ml clarified supernatant added to 7.5x 4x 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 pbl 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 ptk1. 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 ptk1 subcloned into pAc52 was 1 used. Other constructs were previously described (Davidson et al., 2005; Miller and Moon, 1997).

**Cell Culture, Luciferase Reporter Assays, colP 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% CO2 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 transfectected in 6-well plates using Effectene (QIAGEN): 500 ng of pAc-human-LRP6, pPACP-lwg and mt-dsh; 250 ng of pAc5.1-mouse-pltk1, pAc-human-CCNY, LEFT-7-luc (TOPFLASH), and pPACP-LEFT-7; 125 ng pRl128-Ruc (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 m CuSO4 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-LRP6, 100 ng; pCMV-mouse-med, 25 ng; pCS-V5-mouse-pltk1, 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 Na2VO4, 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 (Jueda et al., 2005) was used. For siRNAs, Dharmaco 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., 2006). Cell Cycle Regulation of LRP6 Phosphorylation

Developmental Cell

Cell Cycle Regulation of LRP6 Phosphorylation

Developmental Cell
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 0.3x 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 TOPOFLASH 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. Morpholin antisense oligonucleotides (Gene Tools) were designed to target both Xenopus laevis and tropicalis ccny and ccny1 and have the following sequences: ccny, 5′-CACAGCAGAGGATGTGGTTCCCCAT-3′; ccny1, 5′-GGGAAAGGTACGGGTGGCCG-3′. Lrp6 (Haslerr et al., 2007) and ccdc25β Morpholinos (Ueno et al., 2008) were described previously. Equal amount of MO were injected by adjustment with the standard control MO, where necessary. Doses injected (ng/embryo) were 2–6 (lpc6), 40–80 (ccny/ccny1 mix, 1:1), 80 (ccdc25β); ng/oocyte: 15–30 (ccny/ccny1 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 μM/ml cycloheximide (Sigma) and homogenized in Triton lysin buffer.

In Vitro Kinase Assays

FLAG-LRP6/3C (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 communoprecipitated 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 μl Ci ATP and incubated for 1 hr at 30°C; kinase reaction buffer contained 80 mM Na-β-glycerophosphate, 15 mM MgCl2, 20 mM EGTA, 0.1 mM Na3VO4, 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 MgCl2, 10 mM Na-Pyrophosphate, 10 mM NaF, 5 mM Na3VO4, 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 (LRP5, 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 LRPs/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 Epoxojim (SIGMA) for 3 hr and immunostained as described (Bilic et al., 2007). Endogenous phosphorylated LRPP6 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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REFERENCES


