Tumor-specific pyruvate kinase M2 (PKM2) is instrumental in both aerobic glycolysis and gene transcription. PKM2 regulates G1-S phase transition by controlling cyclin D1 expression. However, it is not known whether PKM2 directly controls cell-cycle progression. We show here that PKM2, but not PKM1, binds to the spindle checkpoint protein Bub3 during mitosis and phosphorylates Bub3 at Y207. This phosphorylation is required for Bub3-Bub1 complex recruitment to kinetochores, where it interacts with Blinkin and is essential for correct kinetochore-microtubule attachment, mitotic/spindle-assembly checkpoint, accurate chromosome segregation, cell survival and proliferation, and active EGF receptor-induced brain tumorigenesis. In addition, the level of Bub3 Y207 phosphorylation correlated with histone H3 at T11, leading to H3-K9 acetylation and transcription of genes, including CCND1 (encoding for cyclin D1) and MYC (Yang et al., 2012b). These findings clearly demonstrate that PKM2 regulates G1-S phase transition by controlling cyclin D1 expression (Yang et al., 2012b). However, whether PKM2 plays a role in regulating mitosis is unknown.

Before cell division, the replicated genome must be accurately segregated to ensure the continued growth and development of the daughter cells (Holland and Cleveland, 2009; Tanaka et al., 2005). Errors in chromosomal segregation can lead to the loss or gain of chromosomes in daughter cells. This condition is called aneuploidy (Holland and Cleveland, 2009). To maintain the fidelity of chromosome segregation, eukaryotes have evolved a control mechanism, often referred to as the cell-cycle checkpoint or the mitotic or spindle assembly checkpoint (SAC), which monitors the status of kinetochore-microtubule (K-MT) attachments and delays anaphase onset until all the chromosomes are correctly aligned on the metaphase plate (Cheeseman and Desai, 2008; Musacchio and Salmon, 2007). SAC component proteins include the evolutionarily conserved Bub1, Bub3, Mad1, Mad2, BubR1 (Mad3 in yeast), Mps1, centromere-associated protein (CENP)-E, and Aurora B proteins (Musacchio and Salmon, 2007). SAC proteins inhibit the ubiquitin ligase activity of the anaphase-promoting complex/cyclosome and the proteasome-mediated destruction of securin and mitotic cyclin B, which blocks separase-dependent cohesion cleavage, the separation of sister chromatids, and cyclin B degradation-dependent mitotic exit (Musacchio and Salmon, 2007). Bub3, Bub1, and BubR1 form cell-cycle-constitutive complexes and are interdependent for kinetochore localization during prometaphase by binding to Blinkin (also known as KNL1, Spc7, Spc105, AF15q14, D40, and CASC5), a member of the conserved KMN (KNL1/Mis12 complex/Ndc80 complex) network of kinetochore proteins (Bolanos-Garcia and Blundell, 2011; Kiyomitsu et al., 2004). Its Y207 phosphorylation is required for Bub3-Bub1 complex recruitment to kinetochores, where it interacts with Blinkin and is essential for correct kinetochore-microtubule attachment, mitotic/spindle-assembly checkpoint, accurate chromosome segregation, cell survival and proliferation, and active EGF receptor-induced brain tumorigenesis. In addition, the level of Bub3 Y207 phosphorylation correlated with histone H3 at T11, leading to H3-K9 acetylation and transcription of genes, including CCND1 (encoding for cyclin D1) and MYC (Yang et al., 2012b). These findings clearly demonstrate that PKM2 regulates G1-S phase transition by controlling cyclin D1 expression (Yang et al., 2012b). However, whether PKM2 plays a role in regulating mitosis is unknown.

Before cell division, the replicated genome must be accurately segregated to ensure the continued growth and development of the daughter cells (Holland and Cleveland, 2009; Tanaka et al., 2005). Errors in chromosomal segregation can lead to the loss or gain of chromosomes in daughter cells. This condition is called aneuploidy (Holland and Cleveland, 2009). To maintain the fidelity of chromosome segregation, eukaryotes have evolved a control mechanism, often referred to as the cell-cycle checkpoint or the mitotic or spindle assembly checkpoint (SAC), which monitors the status of kinetochore-microtubule (K-MT) attachments and delays anaphase onset until all the chromosomes are correctly aligned on the metaphase plate (Cheeseman and Desai, 2008; Musacchio and Salmon, 2007). SAC component proteins include the evolutionarily conserved Bub1, Bub3, Mad1, Mad2, BubR1 (Mad3 in yeast), Mps1, centromere-associated protein (CENP)-E, and Aurora B proteins (Musacchio and Salmon, 2007). SAC proteins inhibit the ubiquitin ligase activity of the anaphase-promoting complex/cyclosome and the proteasome-mediated destruction of securin and mitotic cyclin B, which blocks separase-dependent cohesion cleavage, the separation of sister chromatids, and cyclin B degradation-dependent mitotic exit (Musacchio and Salmon, 2007). Bub3, Bub1, and BubR1 form cell-cycle-constitutive complexes and are interdependent for kinetochore localization during prometaphase by binding to Blinkin (also known as KNL1, Spc7, Spc105, AF15q14, D40, and CASC5), a member of the conserved KMN (KNL1/Mis12 complex/Ndc80 complex) network of kinetochore proteins (Bolanos-Garcia and Blundell, 2011; Kiyomitsu et al., 2004). Its Y207 phosphorylation is required for Bub3-Bub1 complex recruitment to kinetochores, where it interacts with Blinkin and is essential for correct kinetochore-microtubule attachment, mitotic/spindle-assembly checkpoint, accurate chromosome segregation, cell survival and proliferation, and active EGF receptor-induced brain tumorigenesis. In addition, the level of Bub3 Y207 phosphorylation correlated with histone H3 at T11, leading to H3-K9 acetylation and transcription of genes, including CCND1 (encoding for cyclin D1) and MYC (Yang et al., 2012b). These findings clearly demonstrate that PKM2 regulates G1-S phase transition by controlling cyclin D1 expression (Yang et al., 2012b). However, whether PKM2 plays a role in regulating mitosis is unknown.
PKM2 Regulates Tumor Cell Mitosis

To examine whether PKM2 plays a role in mitosis, we synchronized HeLa human cervical cancer cells in the G1 phase with a double-thymidine block and then released the block by removing thymidine for 12 hr. Immunofluorescence analyses showed that PKM2 colocalized with chromatin and CENP-A, a centromere-specific histone H3 variant and a marker of kinetochore localization (Cheeseman and Desai, 2008), primarily in prometaphase (and to a lesser extent in metaphase), but not in interphase (Figure 1A). The observed colocalization was abrogated by expression of PKM2 shRNA (Figure S1A available online). In line with this finding, immunoblotting studies revealed that PKM2 was enriched in chromatin extracts of mitotic cells that were indicated by the mitosis marker phosphohistone H3-S10 (Cheung et al., 2000) (Figure 1B, left panel). PKM2 association with chromatin was also observed in cells treated with nocodazole after a double-thymidine block that arrested the cells at mitosis (Figure 1B, right panel). The amount of chromatin-associated PKM2 was reduced after mitotic exit prompted by removal of nocodazole for 2 hr. These results suggest a role for PKM2 in mitosis progression.

To avoid the effect of PKM2 depletion on G1-S transition and investigate whether PKM2 plays a role in mitosis regulation, we expressed doxycycline-inducible PKM2 shRNA and depleted PKM2 in the mitotic HeLa cells (data not shown) and U87 human glioblastoma (GBM) cells expressing an active EGFRvIII mutant (Figure 1C, left panel), which were released from a double-thymidine block. Flow cytometric analysis showed that about 70% cells were in mitosis and only a small portion of cells were in G0/G1 phase, which may result from unsynchronized PKM2 depletion in heterogeneous GBM cells (some tumor cells having less PKM2 may have earlier depletion than others were arrested at G0/G1 phases) (Figure S1B). PKM2 depletion during mitosis prevented microtubule spindle attachment to kinetochores in metaphase (Figure 1D, top panel) and resulted in abnormal chromosome segregation and lagging chromatids in anaphase (Figure 1D, middle panel) and higher levels of micronuclei in the cells that had exited mitosis (Figure 1D, bottom panel). These PKM2 depletion-induced mitotic defects were rescued by the reconstituted expression of RNAi-resistant wild-type (WT) rPKM2 (Figures 1C, right panel, and 1D). In addition, live-cell time-lapse image analyses showed that PKM2 depletion, which did not significantly affect spindle assembly, still impaired spindle attachment to chromosomes (Figures 1E and S1C). These results indicated that PKM2 is required for correct K-MT attachment and the fidelity of chromosome segregation.

PKM2 phosphorylates histone H3-T111, leading to H3-K9 acetylation (Yang et al., 2012b), which could preclude histone H3-K9 methylation for the recruitment of chromosome passenger complex (CPC) proteins, such as Aurora B, to centromeric DNA for regulating chromosome segregation (Verdaasdonk and Bloom, 2011; Zhang et al., 2010). To test this possibility, we performed ChIP assays with antibodies for H3-pT11, acetylated H3-K9, methylated H3-K4 and H3-K9, and CENP-A and primers specific for centromeric DNA. We found that PKM2 depletion did not affect the levels of H3-T11 phosphorylation, acetylation or methylation of H3-K4 and H3-K9, or CENP-A expression in centromeric DNA regions (Figure S1D), suggesting that PKM2 does not phosphorylate histone H3 across kinetochore regions or affect CENP-A centromere localization. Immunofluorescence analyses showed that PKM2 depletion, which had no effect on the localization of CENP-A, did not alter the localization of other kinetochore proteins, such as CENP-C, CENP-T, and CENP-U, in the interphase and prometaphase of HeLa cells (Figure S1E). The recruitment of CPC proteins, such as Aurora B, to kinetochore regions was also not largely affected (Figure S1F). In contrast, PKM2 depletion, which abrogated the colocalization between PKM2 and the Bub3 and Bub1 complex (Figure S1G), blocked the recruitment of Bub3 and Bub1 to kinetochores during the prometaphase of U87/EGFRvIII (Figure 1F) and HeLa (data not shown) cells without affecting the expression levels of Bub3 and Bub1 as well as CENP-A, CENP-C, CENP-T, and CENP-U and Aurora-B (Figure S1H). The PKM2 depletion-induced defect was rescued by the reconstituted expression of rPKM2 (Figures 1F and S1G). These results indicated that PKM2 is essential for the translocation of Bub3 and Bub1 to kinetochores.

PKM2, but Not PKM1, Interacts with and Phosphorylates Bub3 at Y207

To determine the relationship between PKM2 and the Bub3-Bub1 complex, we synchronized HeLa cells with a double-thymidine block followed with or without nocodazole treatment. Immunoblotting of immunoprecipitated Bub3 with an anti-PKM2 or an anti-PKM1 antibody showed that, in contrast to the constant association between Bub3 and Bub1 (Figure 2A, third panel), Bub3 interacts with PKM2, but not with endogenous (Figure 2A, top two panels) or overexpressed PKM1 (Figure S2A), during mitosis, but not in interphase or after mitosis exit. To examine whether PKM2 directly binds to Bub3 or Bub1, we incubated bacterially purified recombinant GST-Bub3 or GST-Bub1 with purified recombinant His-PKM2 or His-PKM1. We found that PKM2, but not PKM1, binds to Bub3, but not Bub1 (Figure 2B). In line with this finding, coimmunoprecipitation analyses showed that Bub1 depletion did not affect the interaction between PKM2 and Bub3 (Figure S2B,
PKM2 Regulates Tumor Cell Mitosis

Figure 1. PKM2 Is Required for the Fidelity of Chromosome Segregation and Kinetochore Localization of Bub3 and Bub1

(A) HeLa cells in different phases of the cell cycle were immunostained with the indicated antibodies. Nuclei were stained with DAPI (blue).

(B) HeLa cells synchronized by thymidine double block (2 mM) were released for the indicated periods of time (left panel) or for 6 hr, followed by nocodazole (20 ng/ml) treatment for 12 hr (right panel), with or without removal of nocodazole for 2 hr thereafter. Chromatin extracts or cell lysates were prepared.

(C and D) U87/EGFRvIII cells synchronized by thymidine double block (2 mM) were released for the indicated periods of time. Doxycycline (500 ng/ml) was added at the indicated time point to induce PKM2 shRNA or scrambled shRNA as a control expression. U87/EGFRvIII cells with expression of PKM2 shRNA were reconstituted with or without WT rPKM2 expression. Immunoblotting analyses were performed with the indicated antibodies (C). MG132 (25 μM) was added at the indicated time point and incubated with the cells for 1 hr to sustain the cells in metaphase. MG132 were then removed for either 15 min or 2.5 hr to release the cells into anaphase or interphase, respectively. These cells were immunostained with the indicated antibodies. One hundred metaphase cells in each indicated phase of mitosis were analyzed. Data represent the mean ±SD of three independent experiments. The white arrows point to the fragmented chromatin, lagging chromosomes, or micronuclei (D).

(E) U87/EGFRvIII cells expressing GFP-tubulin and mCherry-Histone H2B (for chromosome staining) synchronized by thymidine double block (2 mM) were released for 9 hr. Doxycycline (500 ng/ml) was added with thymidine to induce PKM2 shRNA expression. Live-cell confocal time-lapse images were taken at the indicated time points.

(F) U87/EGFRvIII cells synchronized by thymidine double block (2 mM) were released for 9 hr. Doxycycline (500 ng/ml) was added at the indicated time point in Figure 1C to induce PKM2 shRNA expression. The cells were stained with the indicated antibodies. See also Figure S1.
left panel). In contrast, Bub3 depletion disrupted the binding of PKM2 to Bub1 (Figure S2B, right panel), further supporting that PKM2 directly binds to Bub3.

Mutations of the polar or hydrophobic surface residues of PKM2 R400, L401, P403, L404, D407, P408, T409, and K433 coded by the PKM2-specific exon 10 and expression of these residues in HeLa cells resulted in a loss of the phosphorylation of Bub3 at Y207. This suggests that PKM2 directly binds to Bub3 and phosphorylates it at Y207.
PKM2 Regulates Tumor Cell Mitosis

PKM2 can function as a protein kinase (Gao et al., 2012; Yang et al., 2012b), we tested whether Bub3 is a substrate of PKM2 kinase activity. An in vitro protein kinase assay of recombinant PKM2 or PKM1 mixed with recombinant Bub3 showed that PKM2, but not inactive PKM2 K367M mutant or PKM1, phosphorylated Bub3; Bub3 phosphorylation was detected by anti-phospho-Tyr (Figure 2E), but not anti-phospho-Ser or anti-phospho-Thr (data not shown) antibodies. Notably, this phosphorylation occurred in the presence of PEP, the physiological phosphate group donor of PKM2, but not in the presence of ATP. Liquid chromatography-coupled ion trap mass spectrometry analyses displayed that PKM2 phosphorylated Bub3 at Y207 (Figure S2D). Mutations of Bub3 Y141, Y194, and Y207 into phenylalanines showed that Bub3 Y207F, but not WT Bub3, Bub3 Y141F, or Bub3 Y194F, was resistant to this phosphorylation (Figure S2E), and autoradiography using 32P-labeled PEP was alleviated by cold PEP, whereas ATP did not affect PEP-dependent Bub3 Y207 phosphorylation (Figure S2F). Furthermore, Bub3 Y207 phosphorylation was enhanced by increasing the amount of PKM2 in the reactions (Figure S2G). These results indicated that PKM2 interacts with and phosphorylates Bub3 at Y207 in vitro. The recombinant WT PKM2 in the presence of fructose 1,6-bisphosphate (FBP), which forms a tetramer (Gao et al., 2012), sufficiently phosphorylated Bub3, as does dimerized PKM2 R399E mutant (Gao et al., 2012) (Figure S3H), failed to phosphorylate Bub3 Y207 during mitosis of HeLa (Figure 2K) and U87/EGFRvIII (Figure S3I) cells. In contrast, expression of rPKM2 P408/409A mutant, which phosphorylated histone H3 T11 and STAT3 Y705 but not Bub3 in vitro (Figure S3J), did not affect PKM2-mediated phosphorylation of histone H3 T11 upon EGF treatment and STAT3 Y705 phosphorylation (Gao et al., 2012; Yang et al., 2012b) (Figure S3K). These results indicated that PKM2 specifically phosphorylates Bub3 Y207 during mitosis.

To further support that PKM2 phosphorylates Bub3 Y207 during mitosis, we depleted endogenous Bub3 and reconstituted the expression of RNAi-resistant WT rBub3, rBub3 Y141F, rBub3 Y194F, or rBub3 Y207F in HeLa cells (Figure S3A) or in U87/EGFRvIII cells (Figure S3B). We demonstrated that only rBub3 Y207F was resistant to phosphorylation during mitosis (Figures 2I and S3C; accumulation of cyclin B1 as a marker of mitosis). These results were further supported by immunofluorescence analyses showing that phosphorylated Bub3 Y207 co-localized with CENP-A and was detected in prometaphase, but not in interphase (Figures 2J and S3D). In addition, depletion of endogenous PKM2 and reconstituted expression of WT rPKM2 or rPKM2 P408/409A in HeLa cells (Figure S3E) revealed that expression of rPKM2 P408/409A mutant, but not its WT counterpart, blocked Bub3 Y207 phosphorylation during mitosis (Figures 2J and S3D). These results were further supported by evidences showing that expression of rPKM2 P408/409A mutant, which in contrast to its WT counterpart did not affect its glycolytic enzyme activity (Figure S3F), nuclear translocation (Figure S3G), or protein kinase ability to induce cyclin D1 expression (Yang et al., 2012b) (Figure S3H), failed to phosphorylate Bub3 Y207 during mitosis of HeLa (Figure S3J) and U87/EGFRvIII (Figure S3K) cells. Because PKM2 can function as a protein kinase (Gao et al., 2012; Yang et al., 2012b), we tested whether Bub3 is a substrate of PKM2 kinase activity. An in vitro protein kinase assay of recombinant PKM2 or PKM1 mixed with recombinant Bub3 showed that PKM2, but not inactive PKM2 K367M mutant or PKM1, phosphorylated Bub3; Bub3 phosphorylation was detected by anti-phospho-Tyr (Figure 2E), but not anti-phospho-Ser or anti-phospho-Thr (data not shown) antibodies. Notably, this phosphorylation occurred in the presence of PEP, the physiological phosphate group donor of PKM2, but not in the presence of ATP. Liquid chromatography-coupled ion trap mass spectrometry analyses displayed that PKM2 phosphorylated Bub3 at Y207 (Figure S2D). Mutations of Bub3 Y141, Y194, and Y207 into phenylalanines showed that Bub3 Y207F, but not WT Bub3, Bub3 Y141F, or Bub3 Y194F, was resistant to this phosphorylation (Figure S2E), and autoradiography using 32P-labeled PEP was alleviated by cold PEP, whereas ATP did not affect PEP-dependent Bub3 Y207 phosphorylation (Figure S2F). Furthermore, Bub3 Y207 phosphorylation was enhanced by increasing the amount of PKM2 in the reactions (Figure S2G). These results indicated that PKM2 interacts with and phosphorylates Bub3 at Y207 in vitro. The recombinant WT PKM2 in the presence of fructose 1,6-bisphosphate (FBP), which forms a tetramer (Gao et al., 2012), sufficiently phosphorylated Bub3, as does dimerized PKM2 R399E mutant (Gao et al., 2012) (Figure S3H), suggesting that both dimers and tetramers of PKM2 can phosphorylate Bub3 in vitro.

To test whether PKM2 phosphorylates Bub3 in cells, we synchronized EGFR-overexpressing U87 cells with nocodazole treatment or a double-thymidine block and showed that PKM2 interacted with Bub3 (Figure S2I), and Bub3 Y207 was phosphorylated during mitosis (Figure 2H). In contrast, PKM2 coimmunoprecipitated with β-catenin only in the cells in G1 phase that were serum starved and EGFR treated for 6 hr, but not in mitotic cells (Figure S2J), suggesting that PKM2 regulates the functions of β-catenin and Bub3 in different phases of cell cycle for cell-cycle progression. The cell-cycle phase-dependent regulation of Bub3 by PKM2 was further supported by treatment with cyclin-dependent kinase 1 inhibitor RO-3306, which blocked histone H3 S10 phosphorylation, arrested the cells at G2-M border (Vassilev, 2006), and inhibited the association between PKM2 and Bub3 (Figure S2J). In line with the finding that PKM2 did not interact with Bub1, purified PKM2 did not phosphorylate purified GST-Bub1 (Figure S2K). In addition, 32P-phosphate-metabolic labeling of U87/EGFRvIII cells revealed that PKM2 depletion largely blocked phosphorylation of Bub3, but not Bub1 (Figure S2L).
defects, as reflected by the misalignment of chromosomes in the metaphase plate and defective chromosome segregation represented by the increased incidence of lagging chromosomes and micronuclei in telophase. These results indicated that PKM2-dependent Bub3 Y207 phosphorylation is required for kinetochore recruitment of Bub3 and Bub1, correct K-MT attachments, and proper chromosome segregation.

PKM2-Dependent Bub3 Y207 Phosphorylation Is Required for Recruitment of Bub3 and Bub1 to Kinetochores

Blinkin interaction with Bub1 is required for recruitment of Bub1 to kinetochores (Kiyomitsu et al., 2007). To examine whether Bub3 Y207 phosphorylation regulates the binding of the Bub3-Bub1 complex to Blinkin, we performed a double-thymidine block followed by coimmunoprecipitation analyses with an anti-Blinkin antibody. We showed that Bub3 interacting with phosphorylated Bub3 Y207 during mitosis in HeLa (Figure 4A) and U87/EGFRvIII (Figure S5A) cells with reconstituted expression of WT rPKM2, but not rPKM2 P408T409A. Coimmunoprecipitation analyses showed that PKM2 associated with Bub3 and Bub1, but not Blinkin, during mitosis (Figure S5B), suggesting that PKM2 does not complex with Blinkin at kinetochores.

The essential role of Bub3 Y207 phosphorylation in the regulation of the interaction between Bub3 and Blinkin was further supported by GST pull-down assays, which showed that purified GST-Bub3 interacted with a limited amount of Blinkin from mitotic HeLa (Figure 4B, left panel) and U87/EGFRvIII (Figure S5C) cells with endogenous PKM2 depletion (Figures S3E and S3G). However, incubation of purified WT Bub3, Bub3 Y141F, or Bub3 Y194F, but not Bub3 Y207F, with purified His-PKM2, which phosphorylated Bub3 Y207, significantly enriched the association between Bub3 and Blinkin in HeLa (Figure 4B, left panel) and U87/EGFRvIII (Figure S5C) cells in a manner dependent on the amount of PKM2 and the presence of PEP (Figure 4B, right panel). In addition, coimmunoprecipitation with an anti-Blinkin antibody showed that Blinkin interacted with Bub1, WT rBub3, rBub3 Y141F, and rBub3 Y194F, but not rBub3 Y207F, in HeLa (Figure 4C) and U87/EGFRvIII (Figure S5D) cells with reconstituted expression of these Bub3 proteins (Figures S3A and S3B).

These observations were further supported by immunofluorescence analyses showing that reconstituted expression of rPKM2 P408/T409A blocked the colocalization of Blinkin with Bub3 (Figure 4D, top panel) or Bub1 (Figure 4E, top panel). In addition, Bub3 Y207F, unlike its WT counterpart, failed to colocalize with Blinkin in kinetochores during prometaphase (Figure 4D, middle and bottom panels), and Bub3 Y207F expression blocked recruitment of Bub1 to Blinkin at kinetochores (Figure 4E, middle and bottom panels). In contrast, reconstituted expression of rBub3 Y207F or rPKM2 P408/T409A did not affect the colocalization of Blinkin with CENP-A at the centromere (Figure S5E). These results indicated that PKM2-dependent Bub3 Y207 phosphorylation is required for recruitment of Bub3 and Bub1 to kinetochores to interact with Blinkin.
Mps1 phosphorylates Blinkin or its homologs to recruit Bub1 to Blinkin in a Bub3-dependent manner (London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012). The treatment of U87/EGFRvIII cells with an Mps1 inhibitor (reversine), which inhibited Bub3/Bub1 kinetochore translocation (data not shown), did not affect the interaction between Bub3 and PKM2 or affect PKM2-dependent Blinkin phosphorylation in mitosis (Figure S5F, left panel). In addition, PKM2 depletion did not affect the interaction between Blinkin and Mps1 (Figure S5F, middle panel), and the interaction between Blinkin and Mps1 was not enhanced in U87/EGFRvIII cells compared with that in U87 cells (Figure S5F, right panel). These results suggest that PKM2-dependent Bub3 phosphorylation and Mps1-mediated Blinkin phosphorylation are separate regulatory mechanisms that coordinate and precisely mediate the translocation of the Bub3/Bub1 complex to kinetochores.

PKM2-dependent Bub3 Y207 phosphorylation is required for spindle assembly checkpoint, cell survival, and cell proliferation

Bub3 and Bub1 are required for SAC and delay the onset of anaphase; failure of SAC leads to an accelerated mitosis exit (Bolanos-Garcia and Blundell, 2011). A double-thymidine block and release of U87/EGFRvIII (Figure 5A) and HeLa (Figure S5G) cells with depleted PKM2 or rBub3 and reconstituted expression of WT rPKM2 or rBub3 Y207F, were synchronized by thymidine double block (2 mM) and arrested at mitosis by nocodazole (20 ng/ml) treatment for 9 hr. The cells were then immunostained with the indicated antibodies. The cells in prometaphase were examined. See also Figure S5.
counterparts, also led to an accelerated mitosis exit (Figures 5A, middle and right panels, and S5G), indicating an instrumental role of PKM2-dependent Bub3 phosphorylation in SAC regulation. These observations were supported by immunofluorescence analyses showing that about 80% of the HeLa cells with reconstituted expression of WT rPKM2 or rPKM2 P408T409A (middle panel), or with Bub3 depletion and reconstituted expression of WT rBub3 or rBub3 Y207F (right panel), were synchronized by thymidine double block (2 mM) and released for the indicated periods of time. Immunoblotting analyses were performed with the indicated antibodies. The intensity of H3 pS10 was quantified (right panel). Data represent the mean ± SD of three independent experiments. I indicates interphase; M indicates mitosis.

(B) HeLa cells with PKM2 depletion and reconstituted expression of WT rPKM2 or rPKM2 P408T409A, or with Bub3 depletion and reconstituted expression of WT rBub3 or rBub3 Y207F, were synchronized by thymidine double block (2 mM) and released for 6 hr, followed by nocodazole (20 ng/ml) treatment for 24 hr. Immunofluorescence analyses were performed with an anti-histone H3-pS10 antibody. One hundred cells were analyzed. Data represent the mean ± SD of five independent experiments.

(C and D) HeLa cells with PKM2 depletion and reconstituted expression of WT rPKM2 or rPKM2 P408T409A, or with Bub3 depletion and reconstituted expression of WT rBub3 or rBub3 Y207F, were synchronized by thymidine double block (2 mM) and released for 6 hr, followed by nocodazole (20 ng/ml) treatment for 36 hr. Flow cytometric analyses of mitotic (C) and apoptotic cells (D) were performed. Data represent the mean ± SD of five independent experiments.

(E) HeLa cells (2 × 10⁴) with PKM2 depletion and reconstituted expression of WT rPKM2 or rPKM2 P408T409A, or with Bub3 depletion and reconstituted expression of WT rBub3 or rBub3 Y207F, were plated and counted 7 days after seeding in DMEM with 2% BSA. Data represent the mean ± SD of three independent experiments. See also Figures S5 and S6.

Figure 5. PKM2-Dependent Bub3 Y207 Phosphorylation Is Required for Spindle Assembly Checkpoint, Cell Survival, and Cell Proliferation

(A) U87/EGFvIII cells with PKM2 depletion and reconstituted expression of WT rPKM2 (left panel) or rPKM2 P408T409A (middle panel), or with Bub3 depletion and reconstituted expression of WT rBub3 or rBub3 Y207F (right panel), were synchronized by thymidine double block (2 mM) and released for the indicated periods of time. Immunoblotting analyses were performed with the indicated antibodies. The intensity of H3 pS10 was quantified (right panel). Data represent the mean ± SD of three independent experiments. I indicates interphase; M indicates mitosis.

(B) HeLa cells with PKM2 depletion and reconstituted expression of WT rPKM2 or rPKM2 P408T409A, or with Bub3 depletion and reconstituted expression of WT rBub3 or rBub3 Y207F, were synchronized by thymidine double block (2 mM) and released for 6 hr, followed by nocodazole (20 ng/ml) treatment for 24 hr. Immunofluorescence analyses were performed with an anti-histone H3-pS10 antibody. One hundred cells were analyzed. Data represent the mean ± SD of five independent experiments.

(C and D) HeLa cells with PKM2 depletion and reconstituted expression of WT rPKM2 or rPKM2 P408T409A, or with Bub3 depletion and reconstituted expression of WT rBub3 or rBub3 Y207F, were synchronized by thymidine double block (2 mM) and released for 6 hr, followed by nocodazole (20 ng/ml) treatment for 36 hr. Flow cytometric analyses of mitotic (C) and apoptotic cells (D) were performed. Data represent the mean ± SD of five independent experiments.

(E) HeLa cells (2 × 10⁴) with PKM2 depletion and reconstituted expression of WT rPKM2 or rPKM2 P408T409A, or with Bub3 depletion and reconstituted expression of WT rBub3 or rBub3 Y207F, were plated and counted 7 days after seeding in DMEM with 2% BSA. Data represent the mean ± SD of three independent experiments. See also Figures S5 and S6.
PKM2 Regulates Tumor Cell Mitosis

To determine whether our finding has general applications, we depleted expression of PKM2 in MDA-MB-231 human breast cancer cells, DU145 human prostate cancer cells, A549 human lung adenocarcinoma cells, PAN-C1 human pancreatic cancer cells, and SW480 human colon adenocarcinoma cells. As shown in Figure S5I, Bub3 Y207 was phosphorylated during mitosis, and this phosphorylation was significantly blocked by PKM2 depletion, which was accompanied by an increase in the percentage of aneuploid cells.

PKM2 is highly expressed during embryonic development (Vander Heiden et al., 2009). To determine whether the function of PKM2 in mitosis is also shared by rapidly proliferating embryonic cells, we depleted PKM2 or Bub3 from immortalized mouse embryonic fibroblasts (MEFs) and reconstituted their expression with WT rPKM2, rPKM2 P408/T409A, WT rBub3, or rBub3 Y207F (Figure S5A). Figure S5B shows Bub3 Y207 was phosphorylated in mitosis, and this phosphorylation was blocked by rPKM2 P408/T409A expression (Figure S5C). In addition, expression of rPKM2 P408/T409A and rBub3 Y207F blocked Bub3 recruitment to the kinetochores (Figure S5D). In addition, a significantly higher fraction of the cells expressing these mutants had DNA content greater than 4N (left panel), underwent apoptosis (middle panel), and had inhibited cell proliferation (right panel) compared with their WT counterparts (Figure S5E). Expression of EGFRVIII in MEFs, which increased PKM2 expression (Figure S5F); the binding of PKM2 to Bub3; Bub3 Y207 phosphorylation (Figure S5G); and cell proliferation (Figure S5H, right panel) resulted in more dramatic defects in cell mitosis and cell proliferation upon reconstituted expression of rPKM2 P408/T409A and rBub3 Y207F than did expression of these mutants in MEFs without EGFRVIII expression (Figures S5A and S5E). These results indicate that PKM2 is instrumental in controlling the mitosis progression of tumor cells and rapidly proliferating MEFs and strongly suggest that tumor cells with enhanced PKM2 expression have increased dependence on PKM2’s regulation on mitosis.

In agreement with the finding that rPKM2 P408/T409A mutant did not affect glycolytic enzyme activity in vitro, we did not detect a significant difference in glucose uptake (Figure S6H) or lactate production (Figure S6I) from the cells with reconstituted expression of rPKM2 P408/T409A and rBub3 Y207F before they entered mitosis. However, expression of both rPKM2 P408/T409A and rBub3 Y207F resulted in decreased glucose uptake and lactate production in the cells after exit from cell mitosis and division, indicating that aneuploid cells with altered gene expression can have inhibited aerobic glycolysis.

To determine whether the role of PKM2 in the regulation of Bub3 is evolutionarily conserved, we examined whether the FBP-regulated isoform of pyruvate kinase CDC19 (Pyk1) in Saccharomyces cerevisiae (Boles et al., 1997) has a similar function to that of human PKM2. Pyk1, which does not contain P408/T409, failed to phosphorylate recombinant human Bub3 in vitro (Figure S6J). In addition, reconstituted expression of Pyk1 and human rPKM2 P408/T409A in PKM2-depleted U87/EGFRVIII cells resulted in a similar rate of segregation defect (Figure S6K). These results strongly suggest that Bub3 regulation by PKM2 is a late-evolving phenomenon for controlling cell mitosis.

Bub3 Y207 Phosphorylation Is Required for Tumorigenesis
To determine the role of PKM2-dependent Bub3 Y207F phosphorylation in brain tumor development, we intracranially injected endogenous PKM2- or Bub3-depleted U87/EGFRVIII cells with reconstituted expression of WT rPKM2, rPKM2 P408/T409A, WT rBub3, or rBub3 Y207F into athymic nude mice. U87/EGFRVIII cells expressing WT rPKM2 or WT rBub3 elicited rapid tumorigenesis (Figure 6A). In contrast, rPKM2 P408/T409A and rBub3 Y207F expression significantly inhibited EGFRVIII-driven tumor growth, prolonged mouse survival (Figures 6A and 6B), and promoted tumor cell apoptosis (Figure 6C). Similar tumorigenesis results were obtained by using GSC11 human primary GBM cells with endogenous Bub3 depletion and reconstituted expression of WT rBub3 or rBub3 Y207F (Figures S7A and S7B). These results indicated that PKM2-dependent Bub3 Y207 phosphorylation is instrumental in EGFR-promoted tumor development.

Bub3 Y207 Phosphorylation Positively Correlates with the Level of H3-S10 Phosphorylation in Human Tumor Specimens
Bub3 Y207 phosphorylation correlates with H3-S10 phosphorylation during mitosis (Figure 2H). To further define the clinical relevance of our finding that PKM2 phosphorylates Bub3 Y207, we used immunofluorescence analyses to examine the levels of Bub3 Y207 phosphorylation and H3-S10 phosphorylation in serial sections of 50 human primary GBM specimens (World Health Organization grade IV). The antibody specificities were validated by using specific blocking peptides (Figure S2E). As shown in Figure 7A, Bub3 Y207 phosphorylation colocalized with H3-S10 phosphorylation. In addition, the levels of Bub3 Y207 and H3-S10 phosphorylation were correlated. Quantification of the staining showed that these correlations were significant (Figure 7B; r = 0.78, p < 0.0001).

In addition, colocalization of Bub3 Y207 phosphorylation with H3-S10 phosphorylation and correlation of the levels of Bub3 Y207 and H3-S10 phosphorylation were also observed in 50 human lung adenocarcinoma specimens (Figures S7C and S7D; r = 0.71, p < 0.001). Furthermore, brain tumor patients whose tumors had low Bub3 Y207 phosphorylation (15 cases) had a median survival duration of 69.8 weeks; those GBM patients whose tumors had high levels of Bub3 Y207 phosphorylation (35 cases) had a significantly lower median survival duration of 40.5 weeks (Figure 7C). These results support a role for PKM2-dependent Bub3 Y207 phosphorylation in the clinical behavior of human GBM and lung adenocarcinoma and reveal a relationship between Bub3 Y207 phosphorylation and the mitotic progression of tumor cells.

DISCUSSION
The mitotic checkpoint is a major cell-cycle control mechanism that guards against chromosome missegregation and the subsequent production of aneuploid daughter cells (Holland and Cleveland, 2009). PKM2 plays a key role as a cytosolic glycolytic enzyme in the regulation of cancer cell glycolysis. PKM2 also processes important nuclear functions and plays a critical role in mitosis.
in regulating gene transcription by phosphorylating histone H3 (Yang et al., 2011, 2012b) and functioning as a transcriptional co-activator (Gao et al., 2012; Luo et al., 2011; Yang et al., 2011). However, whether PKM2 directly regulates cell-cycle progression by mediating the mitosis process is not known. In this report, we demonstrate that PKM2 interacts with Bub3 and phosphorylates Bub3 Y207, which leads to the recruitment of the Bub3-Bub1 complex to Blinkin in kinetochores, precise control of kinetochore-spindle microtubule attachment and SAC, and, subsequently, accurate chromosome segregation and proliferation of tumor cells.

Aneuploidy is associated with cancer and tumorigenesis; however, it also adversely affects cell proliferation and the growth of organisms owing to the gain or loss of hundreds or thousands of genes and the disruption of a large array of cellular activities. Thus, aneuploidy can either promote or suppress tumor formation, depending on the genetic and cellular context, including the specific genes on the abnormal chromosome, the extent of the aneuploidy, the already accumulated genetic errors, and specific features unique to the cell type (Holland and Cleveland, 2009). In mammals, complete inactivation of the mitotic checkpoint leads to massive chromosome missegregation, cell death, and early embryonic lethality (Dobles et al., 2000; Michel et al., 2001; Williams et al., 2008). Depleting the SAC proteins BubR1 or Mad2 or inhibiting BubR1 kinase activity causes apoptotic cell death in human cancer cells (Kops et al., 2004). Depletion of Bub1, Bub3, or Blinkin leads to chromosome missegregation and mitosis defects (Kiyomitsu et al., 2007; Logarinho and Bousbaa, 2008). Consistent with the critical role of SAC proteins in mitosis, Bub1-null mice are embryonically lethal (Jeganathan et al., 2007). Similarly, Bub3-null embryos accumulate mitotic errors in the form of micronuclei, chromatin bridging, lagging chromosomes, and irregular nuclear morphology that result in failure to survive. Bub3-null embryos treated with a spindle-depolymerizing agent fail to arrest in metaphase and show an increase in mitotic defects (Kalitsis et al., 2000). In line with

Figure 6. Bub3 Y207 Phosphorylation Is Required for Tumorigenesis

(A) A total of $5 \times 10^5$ U87/EGFRvIII cells with PKM2 depletion and reconstituted expression of WT rPKM2 or rPKM2 P408/T409A (left panel), or with Bub3 depletion and reconstituted expression of WT rBub3 or rBub3 Y207F (middle panel), were intracranially injected into athymic nude mice. The mice were sacrificed and examined for tumor growth. Hematoxylin and eosin (H&E)-stained coronal brain sections show representative tumor xenografts. Tumor volumes were measured by using length (a) and width (b) and calculated using the following equation: $V = \frac{ab^2}{2}$. Data represent the mean ± SD of seven mice (right panel).

(B and C) A total of $5 \times 10^5$ U87/EGFRvIII cells with PKM2 depletion and reconstituted expression of WT rPKM2 or rPKM2 P408/T409A, or with Bub3 depletion and reconstituted expression of WT rBub3 or rBub3 Y207F, were intracranially injected into athymic nude mice. The mouse survival times were recorded. Data represent the mean ± SD of five mice from each group.

* $p < 0.01$: statistically significant value in relation with tumor cells expressing WT rPKM2 or WT rBub3 (C). See also Figure S7.
these evidences of the essential roles of Bub1, Bub3, and Blinkin in kinetochore-spindle microtubule attachment and mitotic checkpoint, we found that reconstituted expression of the Bub3-binding defect mutant of PKM2 in endogenous PKM2-depleted cancer cells displayed a similar mitotic defect, aneu- ploid formation, and cell apoptosis. Our findings support that PKM2-dependent Bub3 Y207 phosphorylation regulates the mitotic functions of the Bub3-Bub1-Blinkin complex and governs the integrity of chromosome segregation, cell survival, and cell proliferation.

Pyk1, a yeast homolog of human PKM2, does not contain P408/T409 for interacting with Bub3 and failed to phosphorylate human Bub3 and rescue the defect induced by PKM2 depletion in tumor cells. In addition, Bub3 in budding yeast does not conserve Bub3 Y207. These findings strongly suggest that Bub3 regulation by PKM2 is a late-evolving phenomenon for controlling the mitosis of tumor cells and rapidly dividing normal mammalian cells. It was recently shown that the β-propeller of yeast Bub3 is important for the binding of Bub3 to the phosphorylated MELT peptides of Blinkin (Primorac

Figure 7. Bub3 Y207 Phosphorylation Positively Correlates with the Level of H3-S10 Phosphorylation in Human GBM Specimens

(A and B) H&E staining and immunofluorescent staining with anti-phospho-Bub3 Y207 and anti-phospho-H3-S10 antibodies were performed on 50 GBM specimens. Representative photos of two tumors are shown (A). We quantitatively scored the tissue sections by counting positively stained cells in ten microscopic fields. (Pearson product-moment correlation test; r = 0.78, p < 0.0001) (B). Note that some of the dots on the graphs represent more than one specimen (some scores overlapped).

(C) The survival times for 50 GBM patients who received standard adjuvant radiotherapy after surgery, followed by treatment with an alkylating agent (temozolomide in most cases), with low (0–4 staining scores, blue curve) versus high (4.1–8 staining scores, red curve) Bub3 Y207 phosphorylation (low, 15 patients; high, 35 patients), were compared. The table (top) shows the multivariate analysis, indicating the significance level of the association of Bub3 Y207 phosphorylation (p = 0.01792) with patient survival. Empty circles represent deceased patients, and filled circles represent censored (alive at last clinical follow-up) patients.

(D) PKM2, which is upregulated in its expression by activation of EGFR or other oncogenic signaling, binds to and phosphorylates Bub3 at Y207, thereby regulating the binding of Bub3-Bub1 complex to Blinkin at kinetochores, spindle assembly, and mitotic checkpoint in tumor cells. See also Figure S7.
et al., 2013). In addition, mutation of R217 and R239 in the β-propeller largely reduced the interaction between Bub3 and Blinkin, suggesting that the β-propeller is responsible for Bub3’s interaction with Blinkin. In line with this finding, Y207 of mammalian Bub3, which aligns with the F222 of the Bub3 yeast homolog, is in the interface involved in the interaction with Blinkin. We demonstrated that the basic binding of Bub3 to Blinkin in tumor cells is substantially enhanced after Bub3 Y207 is phosphorylated by PKM2. These results strongly suggest that oncogenic signaling in mammalian cells regulates the mitosis through the same Bub3-Blinkin interface with a posttranslational modification to increase the binding of Bub3 to Blinkin.

The tumor cells with expression of the active EGFRvIII mutant, which resulted in an increased PKM2 expression (Yang et al., 2012a), had increased dependence on PKM2 during mitotic progression. This strongly suggests that the tumor cells developed their own specific regulatory mechanism for mitosis by regulating the expression of PKM2, which in turn regulated cell-cycle progression via a feedback mechanism. In line with these results, the MEFs, which have lower expression of PKM2 than that in tumor cells, had low rates of defects during mitotic progression under the condition of PKM2 depletion. However, expression of EGFRvIII oncprotein in these fibroblasts increased PKM2 expression and enhanced the interaction between PKM2 and Bub3 and subsequent Bub3 Y207 phosphorylation, which led to increased dependence of the cells on PKM2-regulated mitotic progression. These findings suggest that normal cells, especially adult normal cells with low levels of PKM2 expression, primarily depend on other mechanisms to regulate cell mitosis, which can be abnormally regulated by aberrantly expressed PKM2 upon activation of oncoproteins (Figure 7D).

Abnormally high expression of SAC proteins such as MAD2, kinetochore component HEC1, and PKM2 is common in human cancers, and elevated levels of these proteins are often associated with a poor prognosis (Holland and Cleveland, 2000a; Mazurek, 2007; Yang et al., 2011). In contrast, reduced expression of SAC proteins such as CENP-E and BubR1, resulting from CENP-E haploinsufficiency and BubR1 heterozygosity, respectively, lowered the tumor incidence in mice (Holland and Cleveland, 2009; Rao et al., 2005). The findings that interruption of Bub3 Y207 phosphorylation results in increased cell apoptosis, inhibition of tumor cell proliferation, and inhibition of EGFR-promoted tumorigenesis, and that Bub3 Y207 phosphorylation correlates with the mitotic progression of tumor cells in GBM and lung cancer specimens, highlight the nonmetabolic function of PKM2 as a protein kinase controlling the mitotic process and may provide a molecular basis for improving the diagnosis and treatment of tumors with upregulated PKM2.

**EXPERIMENTAL PROCEDURES**

**Materials**

Rabbit polyclonal antibodies recognizing Bub3, phospho-Bub3 Y207, PKM1, PKM2, and phosphohistone H3-S10 were obtained from Signalway Antibody (College Park). A polyclonal antibody for cyclin D1 was purchased from Santa Cruz Biotechnology (Santa Cruz).

**Transfection**

Cells were plated at a density of $4 \times 10^5$ per 60 mm dish 18 hr prior to transfection. Transfection was performed using HyFect reagents (Denville Scientific) according to the vendor’s instructions. Transfected cultures were selected with puromycin (5 μg/ml), hygromycin (200 μg/ml), or G418 (400 μg/ml) for 10–14 days at 37°C. At that time, antibiotic-resistant colonies were picked, pooled, and expanded for further analysis under selective conditions.

**Cell Proliferation Assay**

Cells (2 × 10^5) were plated and counted 7 days after seeding in DMEM with 0.5% BSA. Data represent the mean ± SD of three independent experiments.

**Flow Cytometric Analysis**

Cells (1 × 10^5) were fixed in 70% ethanol on ice for 3 hr, spun down, and incubated for 1 hr at 37°C in PBS with DNase-free RNase A (100 μg/ml) and propidium iodide (50 μg/ml). Cells were then analyzed by fluorescence-activated cell sorting.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2013.11.001.

**ACKNOWLEDGMENTS**

We thank Don Cleveland (Ludwig Institute for Cancer Research, UCSD) for the LAP-CENP-T and CENP-U plasmids, Iain M. Cheeseman (Whitehead Institute for Biomedical Research, MIT) for the mCherry–H2B plasmid, and Katsumi Kitagawa (St. Jude Children’s Research Hospital) for His-Bub3 and Bub1 plasmids. We thank Lewis Cantley and Costas Lyssiotis for their insightful suggestions and Dawn Chalae for her critical reading of this manuscript. This work was supported by National Cancer Institute grants 2R01CA109035 (Z.L.), 1R01CA168603 (Z.L.), and CA16672 (Cancer Center Support Grant); research grants (RP110252 and RP130389; Z.L.) from the Cancer Prevention and Research Institute of Texas (CPRIT); an American Cancer Society Research Scholar Award (RSG-09-277-01-CSM; Z.L.); the James S. McDonnell Foundation 21st Century Science Initiative in Brain Cancer Research Award (220020318; Z.L.); and the Odyssey Fellowship from The University of Texas MD Anderson Cancer Center (Y.J.).

Received: May 17, 2013
Revised: September 27, 2013
Accepted: October 28, 2013
Published: December 5, 2013

**REFERENCES**


**EXPERIMENTAL PROCEDURES**

**Materials**

Rabbit polyclonal antibodies recognizing Bub3, phospho-Bub3 Y207, PKM1, PKM2, and phosphohistone H3-S10 were obtained from Signalway Antibody (College Park). A polyclonal antibody for cyclin D1 was purchased from Santa Cruz Biotechnology (Santa Cruz).
PKM2 Regulates Tumor Cell Mitosis


