# The v-Jun point mutation allows c-Jun to escape GSK3-dependent recognition and destruction by the Fbw7 ubiquitin ligase

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## Summary

**The c-Jun and c-Myc oncogenic transcription factors are highly unstable proteins due to polyubiquitination. Similar to c-Myc, we report here that phosphorylation of c-Jun by GSK3 creates a high-affinity binding site for the E3 ligase Fbw7, which targets c-Jun for polyubiquitination and proteasomal degradation. In keeping with this, we found that c-Jun levels were inversely related to GSK3 activity in mammalian cells that had entered the cell cycle. Importantly, phosphorylation of c-Jun by GSK3 requires a priming phosphorylation event at Ser-243. Ser-243 is mutated to phenylalanine in v-Jun and allows it to escape recognition by Fbw7. These findings explain the enhanced stability and oncogenicity of v-Jun relative to its cellular counterpart and reveal that GSK3 and Fbw7 coordinately regulate c-Jun and c-Myc.**

## Introduction

The c-Jun oncoprotein, which is a highly unstable transcription factor, can heterodimerize with c-Fos to form AP-1 transcription factor complexes (Shaulian and Karin, 2002). AP-1 has important functions in a wide range of cellular activities including cellular proliferation, transformation, and death. For example, c-Jun and its viral oncogene counterpart, v-Jun, cooperate with Ha-Ras in malignant transformation of murine cells (Weiss and Bohmann, 2004). Additionally, dysregulation of c-Jun has been reported in many types of human cancer (Karin, 1995; Shaulian and Karin, 2002; Weiss and Bohmann, 2004). Hence, cellular c-Jun activity must be very tightly regulated, and there is evidence that it is controlled by both transcriptional and posttranslational mechanisms (Shaulian and Karin, 2002; Weiss and Bohmann, 2004).

c-Jun shares many similarities with the oncoprotein c-Myc. Both proteins play critical roles in driving cells through S phase, and aberrant overexpression of either c-Jun or c-Myc leads to apoptosis. Additionally, both are the products of immediate-early genes whose mRNAs transiently peak at the beginning of the G0-G1 transition and then rapidly decrease. Moreover, the stability of both c-Jun and c-Myc are tightly regulated after cell cycle reentry because of polyubiquitination (Gregory and Hann, 2000; Salghetti et al., 1999; Salvat et al., 1998; Treier et al., 1994). The transforming ability of v-Jun, the viral counterpart of c-Jun, is due at least partly to increased stability (Weiss and Bohmann, 2004). Several groups showed that c-Myc is polyubiquitinated in a GSK3-dependent manner by an SCF complex containing the F box protein Fbw7 (also called hCdc4, Sel-10, and Fbxw7) (Moberg et al., 2004; Welcker et al., 2004; Yada et al., 2004). How c-Jun polyubiquitination is achieved following mitogenic stimulation, and how this process is subverted by v-Jun, are not understood. This prompted us to examine whether c-Jun and c-Myc are targeted for degradation in the same manner and whether this process is altered by the mutations present in v-Jun.

Notably, a recent paper by Nateri and coworkers reported that phosphorylation of the c-Jun N terminus by JNK in neuronal cells targets c-Jun for polyubiquitination by Fbw7 (Nateri et al., 2004). However, this conclusion seemingly contradicts earlier studies demonstrating that JNK phosphorylation protects c-Jun from ubiquitination and subsequent degradation (Fuchs et al., 1997; Musti et al., 1997). Here, we show that phosphorylation of the c-Jun C terminus by GSK3 leads to Fbw7 binding and destruction of c-Jun. Moreover, this site is

### SIGNIFICANCE

**Dysregulation of the c-Jun transcription factor can transform cells. The avian viral oncogene v-Jun lacks the c-Jun δ domain and has two missense mutations at Ser-243 and Cys-269. We found that mutation of Ser-243, which is important for phosphorylation of c-Jun by GSK3, prevents c-Jun from being earmarked for destruction by the Fbw7 ubiquitin ligase complex. Our results assign a biological significance to the v-Jun point mutation and underscore the importance of the Fbw7-c-Jun interaction in the control of cell proliferation. GSK3-dependent Fbw7 binding sites are also present in c-Myc and cyclin E, suggesting that decreased GSK3 activity in cancer cells, such as occurs with increased PI3K/Akt or Wnt signaling, would simultaneously activate c-Jun, c-Myc, and cyclin E.**

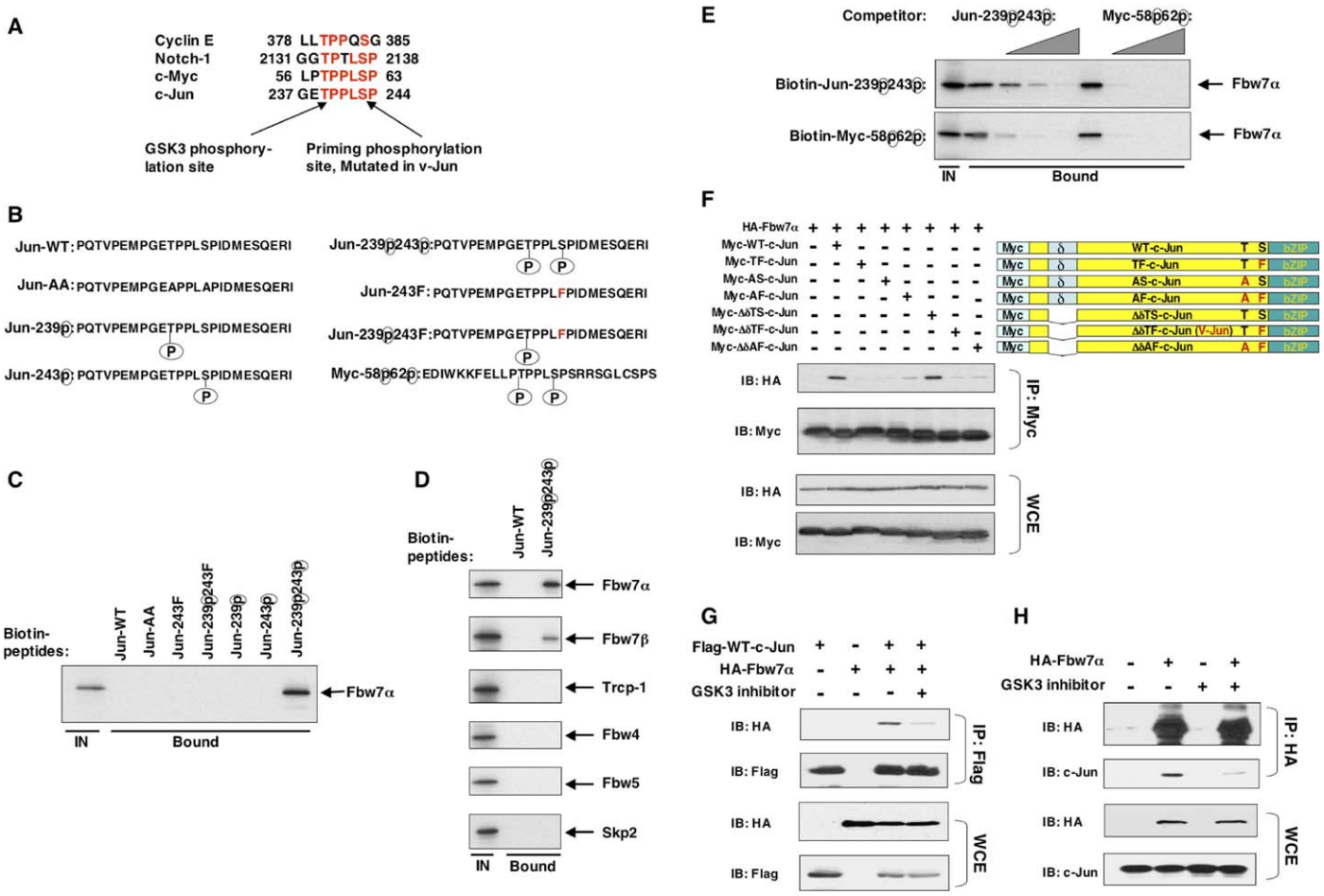


**Figure 1.** Binding of Fbw7 to a c-Jun degron containing phosphothreonine 239 and phosphoserine 243

**A:** Sequence alignment of c-Jun with cyclin E, Notch1, and c-Myc Fbw7 phosphodegrons.

**B:** Sequences of biotinylated c-Jun and c-Myc peptides used in Fbw7 binding assays.

**C–E:** Autoradiograms showing recovery of $^{35}$S-labeled F box proteins bound to indicated biotinylated peptides. IN, input (10% in [C]; 20% in [D] and [E]). In **E**, increasing amounts of nonbiotinylated c-Jun or c-Myc phosphopeptides were added to the binding reactions (5-, 16-, or 50-fold excess, as indicated by the triangles).

**F–H:** Immunoblot (IB) analysis of whole-cell extracts (WCE) and immunoprecipitates (IP) derived from HeLa cells (**F and G**) or 293T cells (**H**) transfected to produce HA-Fbw7α and the indicated Myc-tagged (**F**) or Flag-tagged (**G**) c-Jun proteins. GSK3 inhibitor was added where indicated in **G** and **H**.

destroyed by a v-Jun point mutation, leading to c-Jun stabilization. In contrast, deletion of the JNK docking site within c-Jun, called the δ domain, or elimination of the c-Jun JNK phosphorylation sites, did not affect the interaction between Fbw7 and c-Jun and did not enhance c-Jun stability. In keeping with these considerations, we found that c-Jun levels are inversely correlated with GSK3 activity, but not JNK activity, as resting cells reenter the cell cycle. These findings support that c-Jun and c-Myc are coordinately regulated by GSK3 and Fbw7.

## Results

### c-Jun contains a sequence that is similar to the c-Myc Fbw7 binding site

Using the Prosite Motif Scan Program, we found that c-Jun contains a collinear sequence that is highly similar to the Fbw7 binding sites in c-Myc, cyclin E, and Notch1 (Ye et al., 2004) (Figure 1A). Importantly, the Fbw7 binding site in c-Myc and the putative Fbw7 binding site in c-Jun are highly conserved through evolution (see Figures S1A and S1B in the Supplemental Data available with this article online). Interestingly, both cyclin E (Welcker et al., 2003) and c-Myc (Welcker et al., 2004) must be phosphorylated by GSK3 for efficient recognition by Fbw7, and this c-Jun sequence contains a well-characterized GSK3 site (Thr-239) (Boyle et al., 1991; Morton et al., 2003). Moreover, phosphorylation by GSK3 requires a priming phosphorylation event at position +4 (Doble and Woodgett, 2003), and c-Jun Ser-243 is mutated to phenylalanine in v-Jun (Maki et al., 1987). ERK1 and DYRK1 can phosphorylate Ser-243 in vitro, but whether they do so in vivo is not known (Morton et al., 2003).

### Phosphothreonine 239 and phosphoserine 243 are necessary for interaction of c-Jun with Fbw7 both in vivo and in vitro

To determine the significance of the observation that c-Jun contains an almost identical central phosphorylation domain

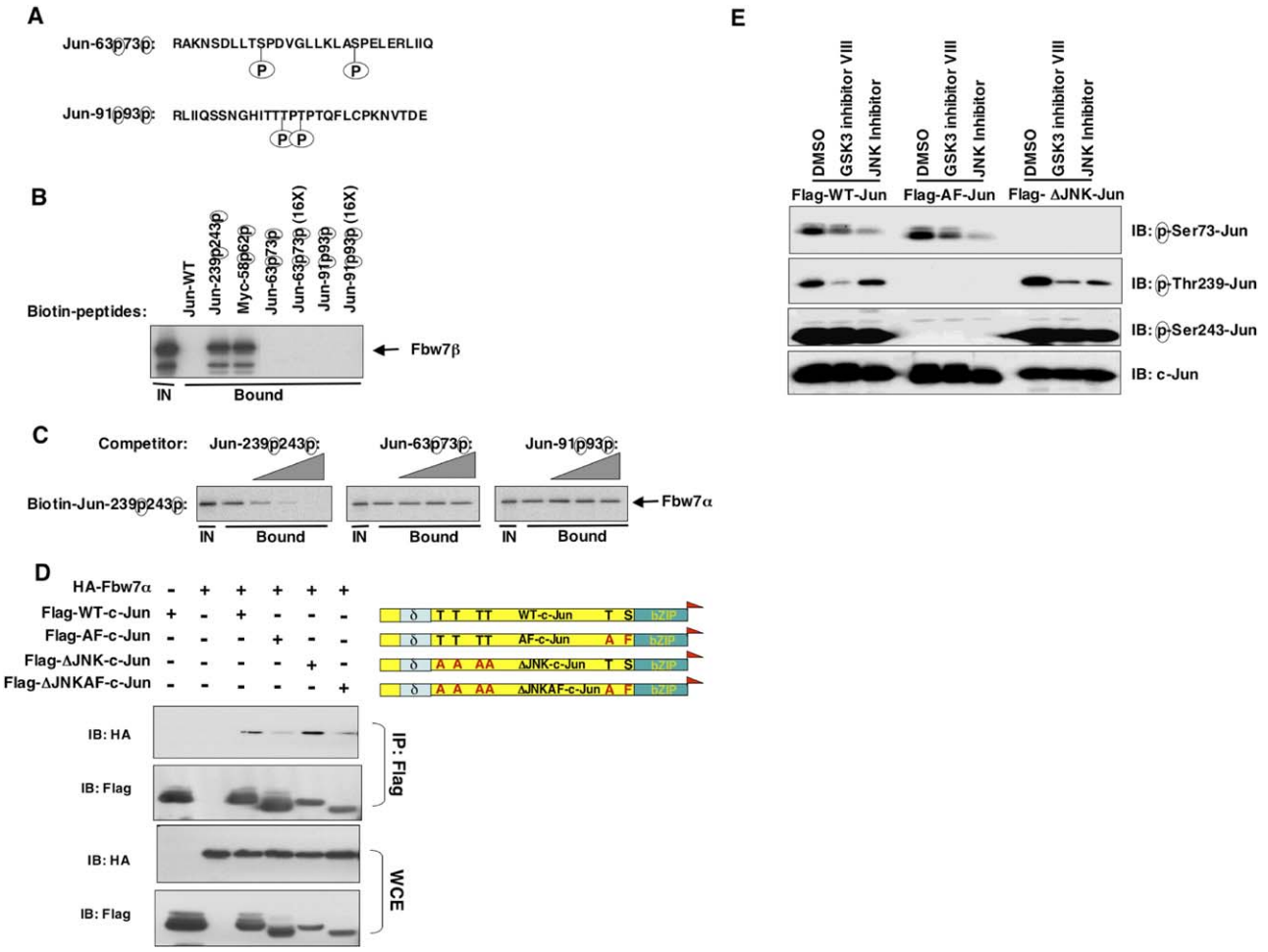


**Figure 2.** Lack of binding of Fbw7 to c-Jun JNK phosphorylation sites

**A:** Sequences of biotinylated c-Jun peptides containing JNK phosphorylation sites.

**B and C:** Autoradiograms showing recovery of $^{35}$S-labeled Fbw7 bound to indicated biotinylated peptides. IN, 20% input. In **C**, increasing amounts of the indicated nonbiotinylated c-Jun phosphopeptides were added to the binding reactions (5-, 16-, or 50-fold excess, as indicated by the triangles).

**D:** Immunoblot (IB) analysis of whole-cell extracts (WCE) and anti-Flag immunoprecipitates (IP) derived from HeLa cells transfected to produce the indicated Flag-tagged c-Jun and HA-Fbw7α proteins. Note that the epitope tag was placed at the C terminus of Fbw7 to avoid interfering with the recognition of the c-Jun N terminus by JNK.

**E:** IB analysis of HeLa cells transfected to produce the indicated Flag-tagged c-Jun proteins and treated with DMSO or the indicated kinase inhibitors (25 μM).

(CPD) as c-Myc (see Figure 1A), we performed Fbw7 binding assays with immobilized synthetic peptides. Fbw7 bound to a peptide corresponding to c-Jun residues 229–253, which spans the putative Fbw7 binding site, provided both Thr-239 and Ser-243 were phosphorylated and the Fbw7 WD40 repeat/substrate binding domain was intact (Figures 1B and 1C and Figures S1C and S1D). In particular, Fbw7 did not recognize peptides containing the v-Jun mutation at residue Ser-243, regardless of the phosphorylation status of Thr-239 (Figure 1C). The doubly phosphorylated c-Jun peptide was recognized by all three Fbw7 isoforms (α, β, γ) but not by a panel of unrelated F box proteins (Figure 1D and data not shown). Cross-competition experiments with the homologous c-Myc phosphopeptide indicated that both c-Jun and c-Myc bind to the same site on Fbw7 (Figure 1E). Disruption of Fbw7 binding in these competition assays was specific, because it was not observed with c-Jun-derived phosphopeptides that do not bind to Fbw7 (see, for example, Figure 2C). Consistent with these in vitro studies, Fbw7 bound to c-Jun in reciprocal coimmunoprecipitation experiments after proteasomal blockade unless Thr-239 or Ser-243 was replaced with alanine or phenylalanine, respectively (Figure 1F and Figure S2). In addition, we found that pharmacological inhibition of GSK3 activity blocked the interaction of HA-Fbw7 with exogenously expressed Flag-c-Jun (Figure 1G) as well as with endogenous c-Jun (Figure 1H) in cells treated with the proteasomal inhibitor MG132. To our knowledge, there are no antibodies available that are capable of recognizing endogenous Fbw7.

### JNK phosphorylation is not required for c-Jun to interact with Fbw7

v-Jun lacks a region called the δ domain, which is a JNK docking site, in addition to the S243F and C269S point mutations. Deletion of the δ domain renders the c-Jun transactivation do-

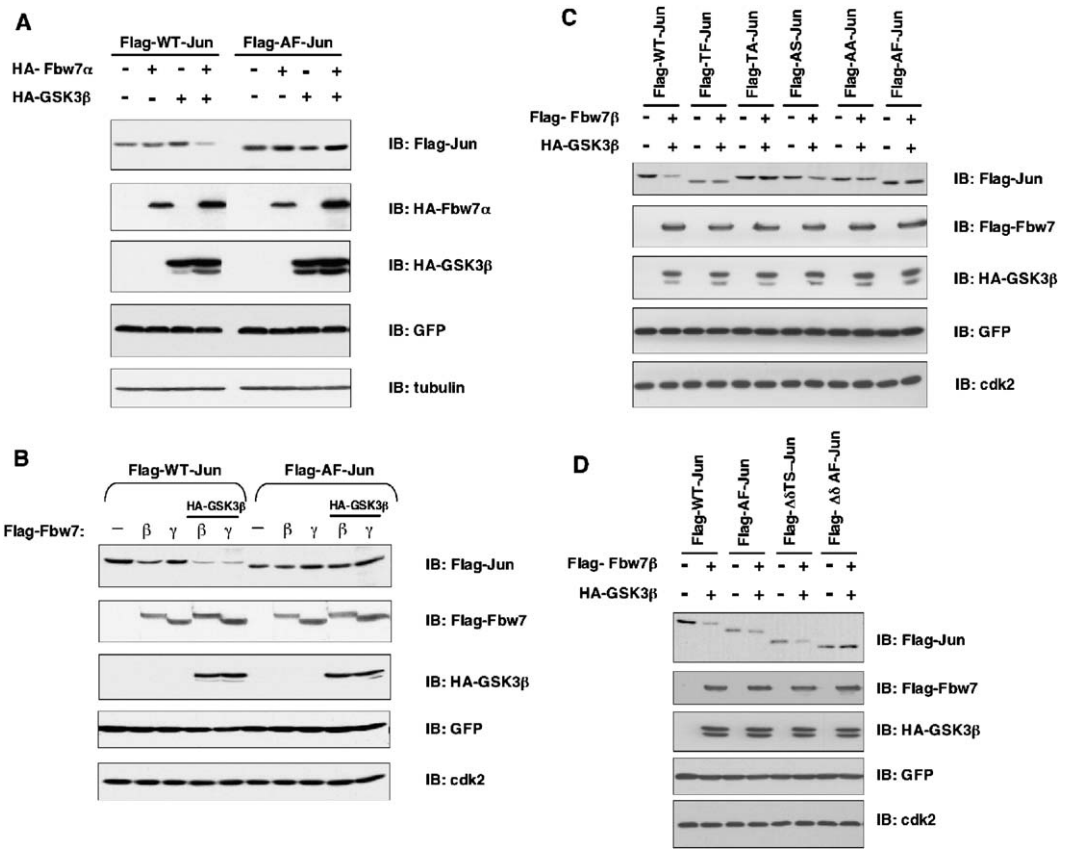


**Figure 3.** Phosphorylation of Thr-239 and Ser-243 is required for Fbw7-mediated c-Jun disappearance

**A–D:** Immunoblot analysis of 293T cells transfected to produce the indicated c-Jun and Fbw7 proteins in the presence or absence of HA-GSK3β. A plasmid encoding GFP was used to control for transfection efficiency.

main constitutively active by preventing the binding of an HDAC3-containing repressor complex (Weiss et al., 2003). The S243F mutation, but not deletion of the δ domain, abrogated Fbw7 binding to c-Jun (Figure 1F). These findings support the idea that Fbw7 binds to c-Jun in GSK3-dependent manner and that this interaction is disrupted by the v-Jun S234F point mutation. While this work was in progress, however, Nateri et al. reported that phosphorylation of N-terminal sites within c-Jun by JNK creates Fbw7 binding sites (Nateri et al., 2004), in apparent disagreement with our finding that the δ domain is dispensable for Fbw7 binding (Figure 1F) and with earlier reports that phosphorylation by JNK protects c-Jun from polyubiquitination (Fuchs et al., 1997; Musti et al., 1997). Moreover, neither of the sites identified by Nateri et al. closely resembles known Fbw7 binding sequences (compare Figures 2A and 1A).

To pursue this further, we repeated our Fbw7 binding assays with the Fbw7 binding c-Jun phosphopeptides described by Nateri et al. Neither peptide, which spans c-Jun JNK phosphorylation sites at residues 63 and 73 or 91 and 93, respectively, bound to Fbw7 under our assay conditions (Figures 2B and 2C and Figure S1E). Moreover, elimination of these phosphorylation sites in the context of full-length c-Jun did not measurably affect Fbw7 binding (Figure 2D) and did not indirectly affect the phosphorylation of c-Jun on Thr-239 and Ser-243 (Figure 2E). Conversely, elimination of the GSK3 sites did not affect the phosphorylation of c-Jun on Ser-63 and Ser-73 (Figure 2E and data not shown). We conclude that binding of Fbw7 to c-Jun occurs primarily via the GSK3-dependent motif.

### Phosphorylation of Thr-239 and Ser-243 is required for Fbw7-mediated c-Jun degradation

Having demonstrated that Fbw7 binds to c-Jun in a GSK3-dependent manner, we next asked whether the binding of Fbw7 could alter c-Jun abundance. As shown in Figure 3A, cotransfection of both Fbw7α and constitutively active GSK3 greatly decreased wild-type c-Jun protein levels but did not affect a c-Jun variant in which both Thr-239 and Ser-243 were mutated (AF-Jun). Similar results were observed with Fbw7β and Fbw7γ (Figure 3B). The potential effects of GSK3 might be underestimated in these assays because the GSK3 priming kinase(s) could be limiting under these conditions. Mutation of either Thr-239 or Ser-243 to alanine allowed c-Jun to escape degradation by Fbw7 (Figure 3C), excluding that the results obtained with AF-Jun were due to conformational changes induced by the phenylalanine substitution at Ser-243. Moreover, deletion of the δ domain, in contrast to mutation of the GSK3 sites, did not allow c-Jun protein to escape Fbw7-mediated protein degradation (Figure 3D). Collectively, these results indicated that phosphorylation of both Thr-239 and Ser-243 is required for Fbw7-mediated c-Jun degradation.

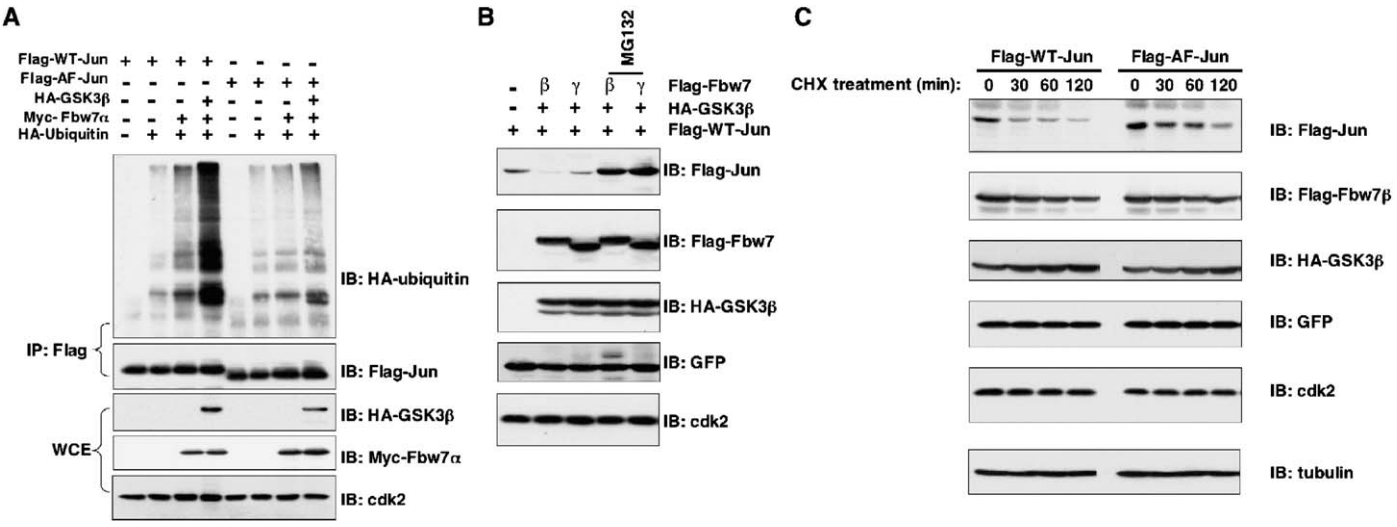


**Figure 4.** GSK3 and Fbw7 promote c-Jun ubiquitination and degradation in vivo

**A:** Immunoblot (IB) analysis of whole-cell extracts (WCE) and anti-Flag immunoprecipitates (IP) of HeLa cells treated with the proteasomal inhibitor MG132 after transfection with the indicated plasmids.

**B and C:** Immunoblot analysis (IB) of 293T cells transfected to produce the indicated Flag-Fbw7, HA-GSKβ, and Flag-c-Jun proteins along with a plasmid encoding GFP. In **B**, MG132 was present for 12 hr where indicated. In **C**, cell extracts were prepared at the indicated time points after the cells were treated with Cycloheximide.

### Promotion of c-Jun ubiquitination by Fbw7 in vivo in a GSK3 phosphorylation-dependent manner

To investigate whether Fbw7 could promote c-Jun ubiquitination in a GSK3-dependent manner, we performed in vivo ubiquitination assays. Cotransfection with Fbw7 led to increased polyubiquitination of wild-type c-Jun, but not AF-Jun (Figure 4A). Furthermore, this effect was magnified in the presence of a constitutively active form of GSK3β. In contrast, elimination of the JNK phosphorylation sites did not affect c-Jun polyubiquitination by Fbw7 (Figure S3). We have been unable to polyubiquitinate wild-type c-Jun with Fbw7 (with or without GSK3) using purified components in vitro under conditions when cyclin E was efficiently ubiquitinated. In pilot experiments, however, we found that Fbw7 produced in wheat germ extract, in contrast to rabbit reticulocyte lysate, did not bind to c-Jun (Figure S1F). Reconstitution of c-Jun polyubiquitination by Fbw7 and GSK3 in vitro will require the identification of the factor(s) responsible for this discrepancy. These factors might, for example, bridge c-Jun and Fbw7 or modify Fbw7. The effect of Fbw7 on c-Jun levels (Figure 3) could be blocked with proteasome inhibitors (Figure 4B and data not shown) and was due to changes in c-Jun half-life, as measured in Cycloheximide chase experiments (Figure 4C), further arguing that the effects of Fbw7 on c-Jun levels is due to polyubiquitination and proteasomal degradation.

### c-Jun protein level inversely correlates with GSK3, but not JNK, activity during cell cycle progression

Next, we monitored the behavior of endogenous c-Jun after serum starvation and release. Human cells contain two *GSK3* genes, called *GSK3*α and *GSK3*β, that are highly similar with respect to sequence and function. It is known that GSK3 is active in serum-starved (quiescent) cells and transiently inactivated by phosphorylation on Ser-21 of GSK3α and Ser-9 of GSK3β in early G1 after serum readdition owing to PI3K/Akt pathway activation (Kim and Kimmel, 2000; Liang and Slingerland, 2003). Hence, Akt activity is inversely related to GSK3 activity. As expected, c-Jun, like c-Myc and cyclin E, was undetectable in serum-starved cells, because these genes are not transcribed under these conditions (Figure 5A). All three proteins were rapidly induced (within 4 hr) after serum refeeding. Importantly, all three proteins began to disappear 8–16 hr after serum readdition, coincident with the activation of GSK3 as determined by a decrease in the inhibitory phosphate (phosphoserine 9) on GSK3β and increased phosphorylation of the GSK3 substrates glycogen synthase (Figure 5A) and Rb-related protein p130 (Figure S4). During this period, cells began to enter S phase (Figure 5B). In contrast, disappearance of c-Jun did not coincide with the appearance of activated, phosphorylated JNK (Figure 5A). JNK, like Akt, is regulated by the Ras pathway and is transiently activated in early G1 phase (Figure 5A; 4 hr time point) (Downward, 1997). Phosphorylation of c-Jun on the JNK sites Ser-63 and Ser-73 preceded the appearance of Ser-243 phosphorylation, which is believed to prime GSK3-dependent phosphorylation of Thr-239. Unfortunately, the previously reported antibodies capable of recognizing the phosphoT239 site in endogenous c-Jun have been exhausted (J. Hastie, personal communication) (Morton et al., 2003). Thus, c-Jun disappearance in this experimental paradigm coincides with GSK3 activation but not JNK activation (See Figure 8).

### Depletion of GSK3 or Fbw7 results in accumulation of c-Jun

To investigate further the contribution of GSK3 and Fbw7 to endogenous c-Jun turnover in vivo, we monitored c-Jun levels

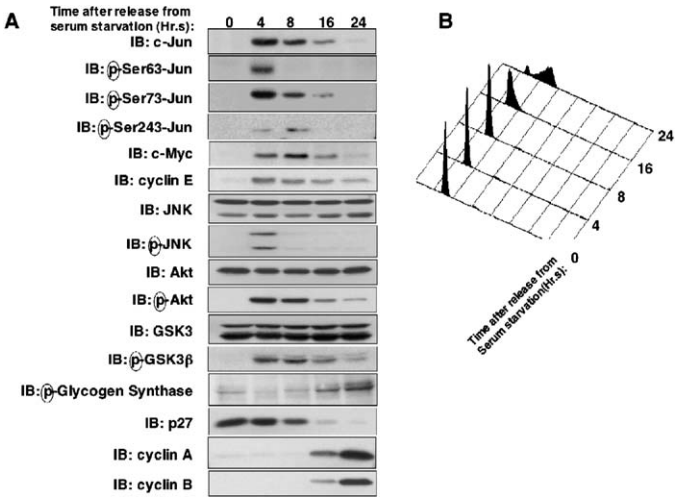


**Figure 5.** Endogenous c-Jun levels inversely correlate with GSK3 during cell cycle progression

Immunoblot (**A**) and FACS (**B**) analysis of T98G cells induced to enter G0 by serum starvation for 72 hr and then released for the indicated time periods.

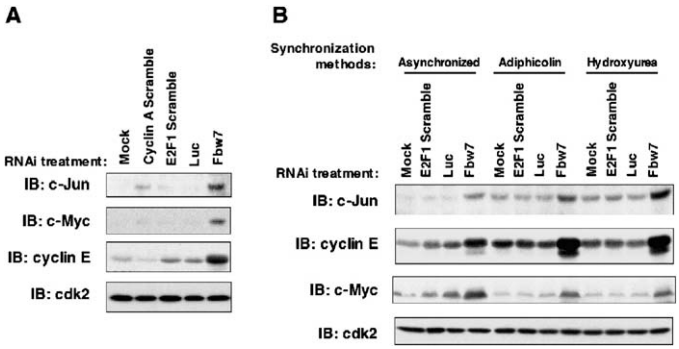


**Figure 6.** Depletion of Fbw7 results in accumulation of c-Jun

Immunoblot analysis of asynchronous (**A**) or synchronized (**B**) HeLa cells transfected with the indicated siRNAs.

after inactivating these proteins. Downregulation of Fbw7 in HeLa cells by RNAi resulted in dramatic increase in the steady state level of c-Jun, c-Myc, and cyclin E (see Figures 6A and 6B), which was accompanied by an increase in the percentage of cells in S phase (Figure S5A). Moreover, the increase in S cell population by Fbw7 depletion is partially due to c-Jun induction, because concurrent downregulation of c-Jun by RNAi treatment greatly reduced S phase population in Fbw7-depleted HeLa cells (see Figures S5B and S5C). Similarly, inhibition of GSK3 with siRNA (Figure 7A) or small molecule GSK3 antagonists (Figures 7B and 7C and Figure S6) led to the accumulation of endogenous c-Jun. The effects of inhibiting Fbw7 or GSK3 were also observed after prior cell synchronization, indicating that the observed increases in c-Jun were not an indirect effect of cell cycle changes induced by these agents (Figures 6B and 7C). In contrast, c-Jun was not increased in cells treated with a JNK inhibitor (Figure 7D and Figure S6D). Phosphorylation of c-Jun on Ser-63 and Ser-73 was, as expected, decreased by the JNK inhibitor but not decreased by the GSK3 inhibitor. Conversely, the GSK3 inhibitor did not decrease Ser-63 or Ser-73 phosphorylation. Pulse-chase experiments using synchronized T98G cells confirmed that endogenous c-Jun half-life was increased with the GSK3 inhibitor and diminished with the JNK inhibitor (Figures 7E and 7F).

## Discussion

Collectively, these results support that Fbw7 regulates c-Jun stability in a GSK3-dependent manner under physiological conditions (see Figure 8). In early G1 phase, growth stimulation leads to a rapid induction of Ras activity and accumulation of PI3K/Akt kinase activities. This leads to phosphorylation of GSK3β on Ser-9 and GSK3α on Ser-21, which inhibits GSK3 kinase activity. As illustrated in Figure 5A, low GSK3 activity at early G1 phase after serum addition correlates with high abundance of c-Jun. In late G1 phase, Akt activity significantly drops due to shutdown of Ras activity. This leads to reactivation of GSK3 activity and a decrease in c-Jun abundance. An inverse correlation between c-Myc abundance and GSK3 activity has also been observed (Sears et al., 2000). These observations support the notion that the timely biphasic control of GSK3 activity downstream of the Ras/PI3K/Akt pathway restricts the accumulation of c-Myc and c-Jun protein to a relatively narrow window in G1. In particular, both proteins are triggered for degradation by GSK3 phosphorylation in later G1 phase once they have accomplished their duties.

The recent suggestion that phosphorylation of c-Jun by JNK triggers c-Jun degradation (Nateri et al., 2004) contradicts early reports indicating that phosphorylation of c-Jun by JNK1 protects it from ubiquitin-mediated degradation (Musti et al., 1997). Recent studies with MEFs deficient in either *Jnk1*, *Jnk2*, or both, are also informative in this regard. From these studies, it appears that JNK1 is the primary kinase capable of phosphorylating c-Jun after serum stimulation and that this phosphorylation event *stabilizes* c-Jun (Sabapathy et al., 2004). In particular, c-Jun is hypophosphorylated, and its levels are *decreased*, in $Jnk1^{-/-}$ MEFs as well as $Jnk1^{-/-}/Jnk2^{-/-}$ MEFs (Sabapathy et al., 2004). It is true that JNK2 can promote c-Jun degradation in serum-starved cells, but this effect does not require JNK2 kinase activity and is not linked to c-Jun phosphorylation (Sabapathy et al., 2004). Moreover, we demonstrated that in several cell lines inhibition of GSK3 activity, but not inhibition of JNK activity, leads to increased c-Jun protein level (Figure 7D and Figure S6D). These results are in agreement with our observation that c-Jun levels are high when Akt and JNK are active and fall during a period when GSK3 becomes active and JNK becomes inactive (Figure 8).

Although these considerations suggest that JNK protects c-Jun from degradation, we cannot formally exclude the possibility that JNK targets c-Jun for destruction in very specific contexts. It should be noted, however, that many of the cell-based experiments reported by Nateri and coworkers used 293T cells (Nateri et al., 2004). Therefore, cell line differences are unlikely to account for our discrepant results. Their results using neuronal cells are consistent with a role of Fbw7 in c-Jun degradation (upon which we agree) and earlier reports showing that JNK is required for c-Jun-dependent apoptosis in such cells but did not prove that JNK targets c-Jun for degradation

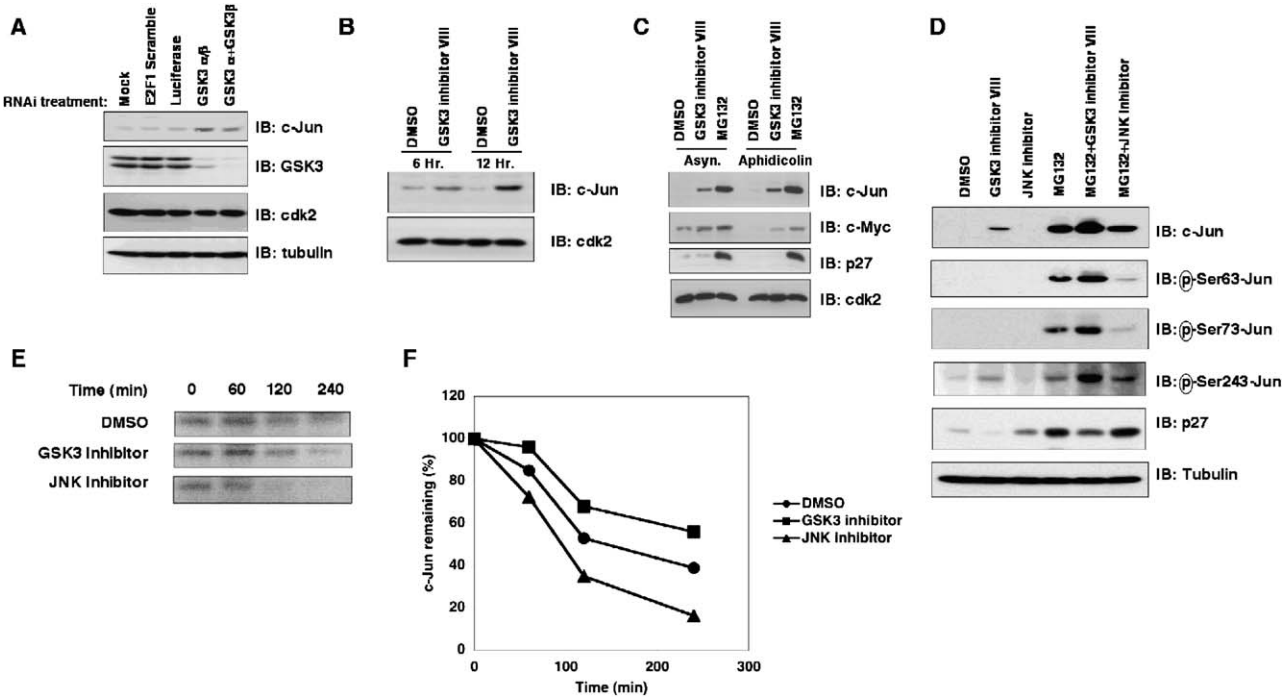


**Figure 7.** Depletion of GSK3 results in accumulation of c-Jun

**A–C:** Immunoblots of HeLa cells transfected with the indicated siRNAs (**A**) or treated with the GSK3 inhibitor VIII (25 μM) (**B and C**). In **C**, GSK3 inhibitor VIII or MG132 was added to asynchronously growing cells or to cells synchronized in S phase by the addition of aphidicolin.
**D:** Immunoblots of T98G cells treated with the GSK3 inhibitor VIII (25 μM) or JNK inhibitor (25 μM).
**E and F:** Pulse-chase analysis of T98G cells. T98G cells were first synchronized at G0 by serum starvation and then refed serum. Four hours later, the cells were treated with GSK3 inhibitor, JNK inhibitor, or vehicle. **E:** Autoradiograms of anti-c-Jun immunoprecipitates obtained at indicated time points during chase. **F:** Quantitation of band intensities in **E**.

in this setting. Nonetheless, it is possible that Fbw7 interacts with the c-Jun JNK phosphorylation sites under specific circumstances not examined here. It is also possible that JNK can, directly or indirectly, affect other c-Jun ubiquitin ligases. In this regard, both Itch (Gao et al., 2004) and COP1 (Wertz et al., 2004) have also been implicated as c-Jun ubiquitin ligases. Ubiquitination of c-Jun in T-lymphocytes by Itch is enhanced after Itch is phosphorylated by JNK (Gao et al., 2004). There is ample precedence for the control of a single protein by multiple ubiquitin ligases that act in different contexts and in response to different signals (Dornan et al., 2004; Lai, 2002).

GSK3 inhibitors have been shown to block neuronal apoptosis mediated by c-Jun (Hongisto et al., 2003), in apparent disagreement with our finding that GSK3 inhibitors upregulate c-Jun in a variety of cells including PC12 cells, which are a standard model for neuronal apoptosis. However, GSK3 has multiple cellular targets, and the effects of GSK3 inhibitors on neuronal apoptosis appear to be complex. In one study, GSK3 inhibitors blocked signals downstream of c-Jun and may have also interfered with upstream apoptotic signals generated by NGF withdrawal (Hongisto et al., 2003).

Our findings, together with an earlier report that phosphorylation of c-Jun by GSK3 on Thr-239 decreases its DNA binding activity, provide a plausible explanation for the enhanced oncogenicity of v-Jun relative to c-Jun (Boyle et al., 1991). However, there have been conflicting results with respect to the contribution of the Ser-243 mutation to transformation by v-Jun. One group reported that introduction of the S243F mutation into c-Jun causes a slight increase in transformation in vitro (Bos et al., 1990) but is sufficient to promote tumorigenesis in vivo (Wong et al., 1992). Another group reported that introduction of the Ser-243 mutation or deletion of the δ domain in c-Jun is sufficient to promote transformation by c-Jun in vitro (Basso et al., 2000). Likewise, we found that the AF mutation increases transformation by c-Jun in soft agar assays (W.W. and W.G.K., unpublished data). We predict that the importance of the Ser-243 mutation to v-Jun-dependent transformation will be influenced by the GSK3 activity of the recipient cells.

The observed inverse correlation of c-Jun, c-Myc, and cyclin E levels with GSK3 activity after cell cycle entry suggests that dual phosphorylation by both priming and GSK3 kinases constitutes a failsafe mechanism, analogous to a two-key lock system, that accurately regulates the degradation of these proteins. Our findings indicate that the S phase-promoting proteins c-Myc, c-Jun, and cyclin E share similar signaling elements, are polyubiquitinated by the same ubiquitin ligase, and therefore exhibit similar periodicity during cell cycle progression. This highly orchestrated cell cycle dependence ensures that these proteins accumulate to levels sufficient to promote S phase entry in response to specific signals and are then quickly degraded to avoid aberrant DNA replication and genomic instability. The requirement for two phosphorylation events might minimize the likelihood that one of these proteins would be prematurely degraded once cells had passed the restriction (R) point in late G1 and become committed to DNA replication.

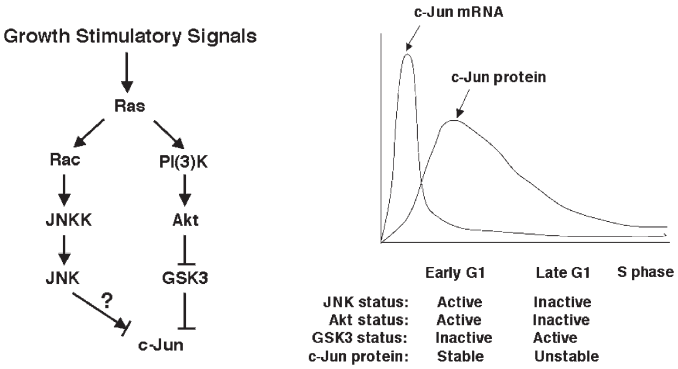


**Figure 8.** Proposed model for pathways that control c-Jun accumulation and degradation during G0 to S phase transition

In early G1 phase, both Akt and JNK activity are activated by Ras after serum stimulation. Activated PI3K/Akt pathway results in inhibitory phosphorylation of GSK3. As cells progress to mid-G1 phase, Ras activity subsides, leading to decreased Akt activity. This permits the reactivation of GSK3, which in turn phosphorylates c-Jun and thereby triggers c-Jun degradation.

Our findings suggest that GSK3 and Fbw7 are master regulators of a number of proteins that play critical roles in cell proliferation and hence would be suspected to play roles in human cancer. Although GSK3 mutations have not been observed in cancer, it is noteworthy that both the Wnt pathway and the PI3K/Akt pathway, which are frequently activated in cancer cells, impinge upon and inhibit GSK3 (Woodgett, 2001). In addition, we note that 4q32, the genetic locus carries the *Fbw7* tumor suppressor gene, is deleted in many types of tumors, and point mutations affecting Fbw7 have been described in breast, colorectal, and endometrial cancers (Rajagopalan et al., 2004; Spruck et al., 2002). Our finding that the GSK3-dependent interaction of Fbw7 with c-Jun is disrupted by the oncogenic v-Jun point mutation provides further genetic evidence that GSK3 and Fbw7 operate to suppress transformation.

## Experimental procedures

### Plasmids

c-*jun* cDNA was amplified by PCR reaction from an image clone (ATCC; catalog no. MGC-3338) with primers that introduced an N-terminal Myc or C-terminal Flag epitope tag and subcloned into pcDNA3 expression vector (Invitrogen). c-Jun mutants were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). HA-tagged Fbw7 α isoform was a kind gift of Prof. Keiichi Nakayama. Flag-tagged Fbw7 β and γ isoforms were obtained from Dr. Bruce Clurman. Fbw4, Fbw5, and HA-tagged GSK3β S9F construct were obtained from Dr. James DeCaprio. The cDNAs used in these studies were all validated by DNA sequencing.

### Antibodies

Anti-c-Jun antibody (9162), anti-phospho-c-Jun (Ser-63) antibody (9261), anti-phospho-c-Jun (Ser-73) antibody (9164), polyclonal anti-Myc epitope antibody (2272), anti-phospho-GSK3β (Ser-9) antibody (9336), anti-Akt antibody (9272), anti-phospho-Akt antibody (4051), anti-JNK antibody (9252), and anti-phospho-JNK antibody (9255) were from Cell Signaling. Anti-Cdk2 antibody (SC-163), anti-p27 antibody (SC-528), polyclonal anti-HA antibody (SC-805), monoclonal anti-Myc epitope (SC-40), anti-cyclin E antibody (SC-247), anti-p130 antibody (sc-317), and anti-c-Jun (H-79) antibody were from Santa Cruz. Anti-tubulin antibody (T-5168), polyclonal anti-Flag antibody (F-7425), monoclonal anti-Flag antibody (F-3165), peroxidase-conjugated anti-mouse secondary antibody (A4416), and anti-rabbit antibody (A4914) were from Sigma. Anti-GSK3 α/β antibody (368662) was from Calbiochem. Anti-GFP antibody (632380) was from Invitrogen. Anti-phospho-glycogen synthase (Ser-641/645) antibody (PB-012) was from Kinasource. Anti-phospho-p130 was a gift from Dr. James DeCaprio. Anti-phospho-c-Jun (Ser-243) and (Thr-239) antibodies were from Dr. James Hastie.

### SiRNAs

*Fbw7* siRNA oligo (sense, 5′-AACCUUCUCUGGAGAGAGAAAUG-3′) and c-*jun* siRNA oligos c-*jun*(1) (sense, 5′-AACGACCUUCUAUGACGAUGC-3′) and c-*jun*(2) (sense, 5′-AAGAACUCGGACCUCCUCACC-3′) were purchased from Dharmacon; *GSK3*α siRNA oligo (6312) and *GSK3* α/β siRNA oligo (6301) were purchased from Cell Signaling. *GSK3*β siRNA oligo was purchased from Ambion (51012).

### Cell culture

Cell culture including synchronization and transfection (plasmid or siRNA) was as described previously (Wei et al., 2004). Where indicated, GSK3β inhibitor VIII (Calbiochem), JNK inhibitor SP600125 (Sigma), MG132 (Calbiochem), or LiCl (Sigma) was added to the cell culture media.

### c-Jun binding assays

Peptides were synthesized by Keck Biotechnology Resource Center of Yale University and dissolved in PBS solution. Peptide (1 μg) was preloaded onto 30 μl of streptavidin agarose and then incubated with 5 μl of $^{35}$S-labeled Fbw7 in vitro translate in 500 μl NETN buffer for 1 hr at 4°C. After five washes with NETN buffer, bound proteins were eluted by boiling in sample buffer, resolved by SDS-PAGE, and detected by autoradiography.

### Protein degradation, polyubiquitination, and pulse-chase analysis

Cells were transfected with a plasmid encoding a Flag epitope-tagged version of the protein of interest along with a plasmid encoding GFP. For half-life studies, Cycloheximide (200 μg/ml; Sigma) was added to the media 48 hr later. At various time points thereafter, cells were removed and protein abundance was measured by immunoblot analysis. For ubiquitination assays, the transfection mix also contained a plasmid encoding HA-ubiquitin. Thirty-six hours after transfection, the proteasome inhibitor MG132 (30 μM) was added. Six hours later, anti-Flag immunoprecipitates were recovered and immunoblotted with anti-HA antibody. For pulse-chase experiments, T98G cells were first serum starved into quiescence for 72 hr. Four hours after readdition of serum, GSK3 or JNK inhibitor was added to the medium for an additional 6 hr. Cells were metabolically labeled for 20 min and then chased exactly as described previously (Wei et al., 2004). Cell lysates were incubated with anti-c-Jun antibody (SC-1694AC, Santa Cruz) overnight at 4°C. Immunoprecipitates were washed six times in NETN buffer and analyzed by SDS-PAGE.

### Supplemental data

The Supplemental Data include six supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/8/1/25/DC1/.

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