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SCF-Mediated Degradation of p100 (NF-κB2): Mechanisms and Relevance in Multiple Myeloma

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Abstract

On the basis of differential analysis of affinity purifications by mass spectrometry, we identified the nuclear factor κB (NF-κB) protein p100 (NF-κB2) as an interactor of the F-box protein FBXW7α. The NF-κB pathway is important for cell growth, differentiation, and survival. p100, which shuttles between the cytoplasm and nucleus, functions as the primary inhibitor of the noncanonical NF-κB pathway by sequestering NF-κB heterodimers in the cytoplasm. in the absence of NF-κB stimulation, the nuclear pool of p100 is constitutively targeted for degradation by FBXW7α, which recognizes a conserved motif that is phosphorylated by glycogen synthase kinase 3 (GSK3). Efficient activation of noncanonical NF-κB signaling depends on the clearance of nuclear p100, either through FBXW7α-mediated degradation or nuclear export mediated by a signal in the C terminus of p100. Upon prolonged stimulation of the NF-κB pathway, p100 is stabilized and retained in the nucleus, contributing to the cessation of noncanonical NF-κB signaling. The molecular mechanism of p100 degradation has implications in multiple myeloma, a disease with constitutive activation of the noncanonical NF-κB pathway. accordingly, expression of a stable p100 mutant, FBXW7α depletion, or chemical inhibition of GSK3 in multiple myeloma cells results in cell death in vitro and in a xenotransplant model. Thus, the FBXW7α-dependent degradation of p100 functions as a prosurvival mechanism through control of NF-κB activity.

Presentation Notes

Slide 1: Science Signaling logo

The slideshow and notes for this Presentation are provided by *Science Signaling* (http://www.sciencesignaling.org).

Slide 2: Title page

This Presentation focuses on the proteolytic regulation of the nuclear factor κB (NF-κB) inhibitor p100 by the SKP1/cullin/F-box protein [SCF, which contains four subunits: a cullin, SKP1 (S-phase kinase-associated protein 1), a RING finger protein (RBX1/HRT1/ROC1), and an F-box protein] ubiquitin ligase FBXW7α (F-box–WD repeat–containing

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protein 7 α) (1). The molecular mechanism of degradation and the relevance of this pathway in multiple myeloma will be discussed.

Slide 3: The hierarchy of the ubiquitin system

The ubiquitin system has evolved in a manner that allows for tremendous diversity and specificity. The ubiquitin system consists of three classes of enzymes: ubiquitin-activating enzymes (E1s or Ubas), ubiquitin-conjugating enzymes (E2s or Ubcs), and ubiquitin ligases (E3s or Ubls). In mammals, there are two E1s, about 30 E2s, and hundreds of E3s, each potentially targeting multiple substrates. Thus, at each step of the ubiquitination pathway, an increased level of specificity is achieved.

Slide 4: The SCF ubiquitin ligase complex

The SCF ubiquitin ligase complex is the prototypical multisubunit really interesting new gene (RING) finger–containing E3 ligase (2, 3). In this complex, cullin 1 (CUL1) is a molecular scaffold that recruits the SKP1–F-box protein–substrate module with its N terminus and the RING finger protein RBX1-E2 module with its C terminus. F-box proteins function as the substrate-targeting subunits, physically interacting with the substrates through specialized protein-protein interaction domains. Once the substrate is positioned in the correct orientation, the E2 (represented by Ubc3 in the diagram) catalyzes the ubiquitylation of the substrate.

Slide 5: The four major families of cullin-RING ubiquitin ligases (CRLs)

There are four major families of cullin-RING ubiquitin ligases (CRLs). These complexes rely on different cullin scaffolds, each of which interacts with different families of proteins specialized for substrate targeting. For instance, CRL3 (CUL3-containing) complexes interact with proteins that contain a broad-complex, tramtrack, and bric-a-brac (BTB) protein-protein interaction domain. CRL4 (CUL4-containing) complexes interact directly with damaged DNA–binding protein 1 (DDB1) and, through DDB1, with DCAF (DDB1- and CUL4-associated factors) proteins. CRL2 (CUL2-containing) and CRL5 (CUL5-containing) complexes interact with suppressor of cytokine signaling (SOCS)–box proteins through the elongin B/elongin C module. Using a common cullin backbone is advantageous because it allows for the creation of various functionally distinct E3s (more than 200) by simply switching the substrate interaction module.

Slide 6: Control of key functions by SCF

For the past 14 years, our laboratory has focused on characterizing the key functions of SCF ubiquitin ligases (CRL1 complexes). Through proteomic approaches, we identified several substrates that are targeted for degradation by SCF. Interestingly, CRL1 complexes function in many different cellular processes, including cell-cycle control, DNA damage responses, and circadian rhythms (4). This talk will focus on a previously undescribed function of FBXW7, a member of the F-box family of proteins.

Slide 7: The best-characterized substrates of FBXW7

The E3 ubiquitin ligase SCF^{FBXW7} targets several substrates for degradation, including cyclin E, c-myc, and notch. Because these proteins are positive regulators of cell proliferation, FBXW7 has been suggested to be a tumor suppressor (5-8). However, this talk will present evidence for a prosurvival function of FBXW7 in the context of diseases featuring elevated NF-κB activity.

Slide 8: Differential purification strategy 1

To identify additional substrates of FBXW7, we took advantage of the published structure of FBXW7 in complex with a substrate (cyclin E) peptide (9). FBXW7 contains a WD40 domain in its C terminus that binds to phosphorylated substrates. This domain contacts the cyclin E peptide with multiple amino acid residues. Specifically, Ser^{462}, Thr^{463}, and Arg^{465} of FBXW7 serve as critical ligase-substrate interaction points. Mutations that change these residues to alanine compromise the binding of FBXW7 to substrates. We used this information to design a purification strategy to identify substrates of FBXW7.

Slide 9: Differential purification strategy 2

By using affinity chromatography methods optimized in our laboratory, we purified complexes containing wild-type (WT) FBXW7α (the nuclear isoform of FBXW7) or FBXW7α in which all three residues of the WD40 domain important for ligase-substrate interaction were mutated to alanines ($FBXW7\alpha^{WD40}$) from human embryonic kidney–293 (HEK-293) cells and analyzed these purifications by using mass spectrometry. The advantage of having the WD40 mutant is that we can compare purifications and select interactors that bind in a substrate-like fashion, that bind only to WT FBXW7α.

Slide 10: Differential purification identifies p100 as a putative FBXW7 substrate

With this purification strategy, we were able to successfully distinguish between potential substrates and other nonsubstrate interactors, such as components of the SCF complex, regulatory proteins, and nonspecific chaperones. We performed both single-tag FLAG-FBXW7α immunopurifications (SIP) and double-tag FLAG–HA (hemagglutin)–FBXW7α immunopurifications (DIP). Positive controls (known substrates), such as cyclin E and notch, were detected only in purifications of WT FBXW7α. Similarly, we found that p100 (NF-κB2) bound only to WT FBXW7α. This protein is an inhibitor of NF-κB and will be the focus of the remainder of this talk.

Slide 11: The NF-κB family of proteins

In order to understand the function of p100, it is important to define the NF-κB family of proteins (10). The NF-κB family is composed of both Rel homology domain (RHD)– and ankyrin repeat domain (ARD)–containing proteins. In general, REL (v-*rel* reticuloendotheliosis viral oncogene homolog) proteins, such as RELA and RELB, are transcriptional activators and bind NF-κB–responsive DNA elements to activate the NF-κB transcriptional program, whereas the inhibitor molecules, such as inhibitor of κB α (IκBα) and IκBβ, feature ARDs. ARD-containing proteins bind NF-κB activators through ARD-RHD interactions and sequester the activators in the cytoplasm, thus inhibiting NF-κB transcriptional regulatory activity. Some proteins, like p100, contain both domains. In the case of p100, the full-length protein is an inhibitor, but p52, a truncated N-terminal fragment generated by a processing event that requires the glycine-rich repeat (GRR), is a transcriptional activator. Here, I will focus on the inhibitory role of full-length p100.

Slide 12: Molecular mechanisms of NF-κB activation and inhibition

The basic mechanisms of NF-κB activation and inhibition have been well characterized. When the pathway is in the inactive state, NF-κB heterodimers are sequestered in the cytoplasm by inhibitory IκBs. Upon activation of the pathway by an extracellular signal, IκBs are eliminated by ubiquitin-dependent proteolysis, and the active transcription factors translocate to the nucleus to promote gene transcription. To turn off the pathway, the IκBs are resynthesized, resequestering the active REL dimers in the cytoplasm and terminating the response.

Slide 13: The noncanonical pathway of NF-κB

NF-κB activity is controlled through two distinct, yet interconnected, pathways that respond to different stimuli. The canonical NF-κB pathway, activated in response to stimuli such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and bacterial endotoxin, is largely dependent on the activity of the IκB kinase (IKK) complex. The noncanonical NF-κB pathway, shown in this slide, is activated in response to developmental factors including LTβ (lymphotoxin beta), CD40L (cluster of differentiation 40), and BAFF (B cell activating factor). This pathway is largely IκB-independent and relies heavily on the inhibitory activity of p100 (11-16). Activation of the noncanonical pathway leads to inhibition of TRAF2 (TNF receptor–associated factor 2)/cIAP (cellular inhibitor of apoptosis)/TRAF3 (TNF receptor–associated factor 3)–mediated degradation of NIK (NF-κB–inducing kinase). Stabilization of NIK leads to hyperphosphorylation of IKKα, which, in turn, phosphorylates p100 on serines 866 and 870. These phosphorylated residues create the docking site for $SCF\beta^{TrCP}$, an SCF complex that includes the substrate recognition protein β-transducin repeat-containing protein (βTrCP), which induces the ubiquitylation and subsequent proteasome-dependent proteolytic processing of inhibitory p100 into activating p52 (17). This event allows the generation of RELB-p52 dimers, which translocate to the nucleus to activate the noncanonical NF-κB transcriptional program.

Slide 14: p100 binds FBXW7α through Ser^{707} and Ser^{711}

We mapped the binding of p100 to FBXW7α. By using proteins produced from a series of p100 C-terminal deletion constructs in HEK-293 cells, we found that FBXW7α contacts a stretch of 20 amino acids (amino acids 700 and 720) in the ARD domain of p100. This region contains a sequence that closely resembles the consensus FBXW7 degradation motif (or degron), which consistently features two phosphorylated residues. Indeed, we identified two serine residues (Ser^{707} and Ser^{711}) in the ARD of p100 that are critical for the FBXW7-p100 interaction. Mutation of these two serines to alanines [$p100^{Ser707/711Ala}$] disrupts the binding of p100 to FBXW7α. Importantly, this binding site is not retained in p52, the processed product of p100.

Slide 15: FBXW7α binds p100 phosphorylated on Ser^{707}

On the basis of our mapping data, we generated an antibody that recognizes p100 only when it is phosphorylated on Ser^{707}. By immunoprecipitating several F-box proteins from whole-cell extracts, we found that p100 binds specifically to FBXW7α, as well as to βTrCP, which has been previously reported. We also found that p100 phosphorylated on Ser^{707} binds only FBXW7α.

Slide 16: FBXW7α and βTrCP bind two different phosphorylated forms of p100

To further clarify the behavior of the phosphorylated forms of p100, we immunoprecipitated FBXW7α, $FBXW7\alpha^{WD40}$, and βTrCP. We found that, whereas FBXW7α specifically binds p100 when p100 is phosphorylated on Ser^{707}, βTrCP only binds to p100 phosphorylated on Ser^{866} and Ser^{870}. These binding properties are mutually exclusive. Thus, these data suggest that FBXW7α and βTrCP target distinct fractions of p100.

Slide 17: GSK3 phosphorylates the p100 degron

For most known substrates of FBXW7, phosphorylation of the degron is performed by glycogen synthase kinase 3 (GSK3) (6). Therefore, we set out to determine whether GSK3 was responsible for phosphorylating p100 on Ser^{707}. Indeed, recombinant GSK3 phosphorylated p100 on Ser^{707} in an in vitro kinase assay. Furthermore, treatment of cells with a GSK3 inhibitor (GSK3i IX) prevented phosphorylation of Ser^{707} (bottom left panel) and prevented the association of p100 with FBXW7α (bottom right panel) in cultured cells.

Slide 18: p100 is ubiquitylated in an $SCF^{FBXW7}\alpha$-dependent manner in vitro

To verify that $SCF^{FBXW7}\alpha$ can support the ubiquitylation of p100, we performed an in vitro ubiquitylation assay using $^{[35]}$S-labeled p100 and $SCF^{FBXW7}\alpha$ purified from insect cells. We found that $SCF^{FBXW7}\alpha$ catalyzed the ubiquitylation of WT p100, but not $p100^{Ser707/711Ala}$, showing that FBXW7α-dependent ubiquitylation of p100 requires an intact degron.

Slide 19: p100 shuttles between the nucleus and cytoplasm

In addition to regulation by processing and degradation, p100 is also regulated at the level of protein localization. Although predominantly visualized in the cytoplasm in unstimulated cells, p100 shuttles continuously between the nucleus and the cytoplasm. In fact, a brief treatment of cells with the exportin/CRM1 inhibitor leptomycin B induced nuclear sequestration of p100. We mapped the nuclear export signal (NES) in p100 to a C-terminal region based on homology with a consensus NES sequence. Mutation of four hydrophobic residues within this signal to alanines resulted in nuclear sequestration of this p100 mutant ($p100^{4\times NES}$).

Slide 20: p100 contains an NLS required for import into the nucleus

In addition to an NES, p100 also contains a nuclear localization signal (NLS) at its N terminus, adjacent to the RHD. p100 relies on the NLS for shuttling into the nucleus because mutation of the NLS sequesters p100 in the cytoplasm, making it insensitive to treatment with leptomycin B.

Slide 21: FBXW7 targets only nuclear p100 for degradation

Through the use of the protein synthesis inhibitor cycloheximide, we discovered that, whereas p100 is largely stable in the cytoplasm, it had a substantially shorter half-life in the nucleus. Upon identification of a NES in p100, we generated a p100 mutant in which four residues in the NES were mutated to alanines ($p100^{4\times NES}$), making this mutant constitutively nuclearly localized. Assessment of the half-life of $p100^{4\times NES}$, with or without additional mutations in the FBXW7 degron ($p100^{4\times NES;Ser707/711Ala}$), revealed that FBXW7 controls the stability of p100 only in the nucleus. Conversely, mutation of the FBXW7 degron had no discernible effect on the stability of the constitutively cytoplasmic p100 mutant ($p100^{NLS}$).

Slide 22: p52 competes with FBXW7 for binding to the ARD of p100

One possible explanation for the difference in stability between the nuclear and cytoplasmic fractions of p100 is the presence or absence of REL protein binding to the p100 ARD. We propose a model wherein REL proteins compete with FBXW7 for binding to the ARD of p100. Indeed, in unstimulated cells, p100 is predominantly cytoplasmic and stable, likely because of binding to REL proteins, which would prevent p100 from binding cytoplasmic FBXW7 (FBXW7β). The data shown here demonstrate that p52 can displace FBXW7α from a p100 complex in vivo.

Slide 23: Conclusions 1

The data shown in the previous slide led us to conclude that cytoplasmic-nuclear shuttling of p100 requires an intact NLS and NES and that the majority of p100 is cytoplasmic because the nuclear pool is constitutively targeted for protein degradation by FBXW7 and GSK3. In contrast, the cytoplasmic pool of p100 is insensitive to FBXW7-mediated degradation.

Slide 24: Constitutive degradation of p100 in the nucleus by FBXW7α is inhibited upon NF-κB activation

To investigate the effect of NF-κB activation on the FBXW7α-mediated degradation of p100, we stimulated *Fbxw7*$^{flox/flox}$ and *Fbxw7*$^{-/-}$ mouse embryonic fibroblasts (MEFs) with antibodies that antagonize the LTβ receptor (α-LTβR). Analysis by subcellular fractionation and Western blot revealed that, before stimulation (time 0), *Fbxw7*$^{-/-}$ MEFs contained higher levels of nuclear p100 than *Fbxw7*$^{flox/flox}$ MEFs. Interestingly, during activation of the noncanonical NF-κB pathway, the generation of p52 closely followed the stabilization of nuclear p100 in *Fbxw7*$^{flox/flox}$ MEFs. This effect was not observable in *Fbxw7*$^{-/-}$ MEFs, leading us to suggest that p52, which is generated and translocated into the nucleus during signaling, competes with FBXW7α to stabilize p100 (shown in Slide 22). This mechanism, along with the de novo synthesis of p100, could be part of a negative feedback loop that terminates the NF-κB response.

Slide 25: Model for the regulation of p100 during NF-κB signaling

We propose a model in which the ubiquitin-proteasome system regulates p100 in two different ways. The first is through inducible processing of p100. IKKα-dependent phosphorylation of p100 results in the binding and ubiquitylation of p100 by SCFβ^{TrCP}, leading to a proteolytic processing event that generates the p52 transcriptional activator (18-20). The second mechanism involves constitutive nuclear degradation of p100. As shown by the results of our studies, FBXW7α and GSK3 facilitate the clearance of nuclear p100. These two regulatory mechanisms act in concert during signaling. Indeed, constitutive degradation of nuclear p100 allows the NF-κB proteins to freely translocate into the nucleus and bind to NF-κB DNA elements upon NF-κB stimulation. Accumulation of NF-κB proteins in the nucleus leads to the stabilization of p100 by displacement of FBXW7α. In turn, the increased abundance of p100 blocks the binding of NF-κB proteins to the NF-κB–responsive promoters, contributing to termination of NF-κB signaling.

Slide 26: Translocation of p52-REL complexes into the nucleus and constitutive degradation of nuclear p100

Upon NF-κB stimulation, the cytoplasmic pool of p100 is removed through βTrCP-dependent inducible processing of p110 to yield p52, which translocates into the nucleus in complex with REL proteins to activate transcription. During the early phase of signaling, constitutive degradation of nuclear p100 by FBXW7α continues, thus allowing NF-κB activation.

Slide 27: p52-REL competition with FBXW7 for binding to p100

Subsequently, p52-REL complexes accumulate in the nucleus. At a threshold concentration in the late phase of signaling, these NF-κB proteins compete with FBXW7α for binding to the ARD of p100 (including newly synthesized p100).

Slide 28: p110 stabilization and termination of NF-κB signaling

The resultant stabilization of p100 in the nucleus terminates the NF-κB response by preventing NF-κB proteins from binding the DNA elements of target gene promoters.

Slide 29: Stabilization of p100 in the nucleus inhibits NF-κB target gene transcription after LTβR activation

In agreement with our model, clearance of nuclear p100 by FBXW7α facilitates the activation of NF-κB–dependent transcription. In this experiment, we either stabilized nuclear p100 by mutating its degron (p100$^{Ser707/711Ala}$) or forced p100 into the nucleus by

mutating its NES ($p100^{4\times NES}$). In both cases, the transcriptional response of NF-κB target genes to LTβR activation was significantly reduced. The expression of a combined nuclear and stable p100 mutant ($p100^{4\times NES;Ser707/711Ala}$) did not further inhibit LTβR-dependent gene transcription. Global transcription remained unchanged, because non–NF-κB responsive genes were unaffected.

Slide 30: Stabilization of p100 in the nucleus inhibits RELB binding at LTβR-responsive gene promoters

To better understand the mechanism of p100 inhibition in the nucleus, we examined the association of RELB with NF-κB elements in the promoters of LTβR-responsive genes. We found, in accordance with the transcriptional data, that expression of a stable p100 mutant ($p100^{Ser707/711Ala}$) or a constitutively nuclear p100 mutant ($p100^{4\times NES}$) prevented the binding of RELB to LTβR-responsive gene promoters. The binding of RELB to LTβR-unresponsive genes did not change.

Slide 31: Conclusions 2

We propose that the molecular mechanisms controlling the abundance of p100 in the nucleus contribute to the initiation and termination of the NF-κB transcriptional program.

Slide 32: Fbxw7 mutations are absent in B cell–derived tumors

Fbxw7 has been described as a tumor suppressor gene. This designation is largely due to inactivating mutations found in several human malignancies, including T cell acute lymphoblastic leukemias (T-ALLs), breast cancer, and other carcinomas (5, 7, 21, 22). Interestingly, mutations in *Fbxw7* have not been detected in B cell–derived tumors. We sequenced the most commonly mutated exons (exons 9 and 10) of *Fbxw7* in 24 multiple myeloma cell lines (MMCLs) and detected no mutations. Other groups have sequenced *Fbxw7* from different B cell–derived tumors and also failed to detect mutations (23-25). Collectively, these data suggest a possible pressure to preserve FBXW7 activity in B cells.

Slide 33: Relevance of NF-κB signaling in stromal-independent multiple myeloma

To investigate a functional role for FBXW7-GSK3–mediated degradation of p100 in the context of B cell malignancies, we moved to a MMCL system. Notably, multiple myeloma is particularly relevant because NF-κB activity is an established marker in the course of the disease (26). In fact, the NF-κB pathway is frequently activated after the primary translocation of multiple myeloma driver genes (like *cyclinD1*). It has been suggested that the initial NF-κB activity stems from signals from the bone marrow stroma, like BAFF and APRIL (a proliferation-inducing ligand). Subsequent genetic alterations of components of the NF-κB pathway allow for the transition of multiple myeloma from a stromal-dependent tumor in the bone marrow to a stromal-independent tumor, free of the bone marrow micro-environment (26).

Slide 34: Mutations activate noncanonical NF-κB signaling in multiple myeloma

Many mutations, particularly in the noncanonical pathway, have been linked to the aberrant activation of NF-κB in multiple myeloma. Activating mutations have been characterized in the genes encoding *Cd40r* (cluster of differentiation 40 receptor), *Ltbr*, *Baff*, and *Nik* (each shown in blue). Deleterious mutations have been found in negative regulators of NF-κB signaling, including *Traf2, Traf3*, and *cIap1* and *cIap2* (each shown in red). C-terminal truncations of p100, which eliminate its IκB activity, have also been identified in multiple myelomas. These genetic alterations result in increased generation of p52 and increased transcription of NF-κB target genes (27, 28).

Slide 35: Depletion of FBXW7 inhibits multiple myeloma cell growth

Because the noncanonical NF-κB pathway plays an important role in multiple myeloma, we investigated whether FBXW7-dependent degradation of p100 is relevant in these tumors. By targeting *Fbxw7* with short hairpin RNAs (shRNAs) in multiple myeloma cell lines, we were able to achieve depletion of the protein, with a consequent increase in the nuclear abundance of p100, particularly in the pool of p100 phosphorylated on Ser^{707} (see red box in Western blot on the right). In addition, we analyzed the rate of proliferation of these multiple myeloma cells and found that FBXW7 depletion reduced their ability to proliferate in culture.

Slide 36: Expression of a p100 mutant resistant to FBXW7-dependent degradation inhibits multiple myeloma cell growth

We next used the p100 degron mutant that is resistant to FBXW7-dependent degradation ($p100^{Ser707/711Ala}$) to determine whether the effects of FBXW7 on the proliferation of multiple myeloma cells are mediated by p100. Expression of $p100^{Ser707/711Ala}$, but not WT p100, impaired the proliferation of cultured multiple myeloma cells. The Western blot on the right shows that both WT and $p100^{Ser707/711Ala}$ undergo normal processing to form p52.

Slide 37: Expression of stable p100 inhibits the growth of myeloma cells xenotransplanted into SCID mice

Furthermore, we examined the effect of expressing a stabilized p100 mutant ($p100^{Ser707/711Ala}$) on the growth of multiple myeloma cells xenotransplanted into severe combined immunodeficient (SCID) mice. Tumor formation was monitored by in vivo imaging of transplanted luciferase-expressing multiple myeloma cells. A representative experiment (left panel) demonstrates that expression of WT p100 has little effect on tumor growth as compared with an empty vector, whereas expression of a stabilized $p100^{Ser707/711Ala}$ significantly impairs tumor growth. In fact, xenografts expressing $p100^{Ser707/711Ala}$ were no longer detectable 28 days after injection. Quantification of tumor growth for three independent xenograft experiments is shown in the right panel.

Slide 38: Forced localization of p100 in the nucleus results in decreased growth of myeloma cells

Similar to stabilization of p100, forced localization of p100 to the nucleus also impaired the growth of multiple myeloma cells. In this experiment, multiple myeloma cells were infected with retroviruses encoding a p100 mutant deficient in nuclear export ($p100^{4\times NES}$). The results of a cell proliferation assay (right panel) reveal a significant decrease in the proliferation of cells expressing $p100^{4\times NES}$ as compared with those expressing WT p100.

Slide 39: Chemical inhibition of the proteasome, cullin-RING ligases, or GSK3 leads to p100 accumulation in the nucleus

Because GSK3 phosphorylates the FBXW7 degron in p100, we tested whether pharmacologic inhibition of this kinase could stabilize p100. There are several potent, specific inhibitors of GSK3 available. We tested three different inhibitors of GSK3 (GSK3is IX, XVI, and XXII) and found that each was capable of stabilizing p100 in the nuclear fraction of multiple myeloma cells (red boxes; upper and middle panels on the right). Interestingly, bortezomib (a proteasome inhibitor approved for the treatment of multiple myeloma) and MLN4924 (a Nedd8-activating enzyme inhibitor that blocks the activity of CRLs) stabilize p100 in the nucleus to a similar extent as GSK3 inhibitors (red box; lower panel on the right).

Slide 40: Chemical inhibition of the proteasome, cullin-RING ligases, or GSK3 is toxic to multiple myeloma cells

We next tested whether treatment with GSK3 inhibitors is cytotoxic in multiple myeloma cell lines. We found that the three different GSK3 inhibitors killed multiple myeloma cells when used at concentrations sufficient to inhibit phosphorylation of p100. MLN4924 demonstrated cytotoxicity at similar concentrations, whereas bortezomib was highly potent in inducing myeloma cell death.

Slide 41: GSK3 toxicity is partially dependent on p100

To determine whether the cytotoxicity of GSK3 inhibition was at least partially dependent on the stabilization of p100, we infected multiple myeloma cell lines with four different lentiviruses encoding shRNAs targeting p100. The efficiency of knockdown is shown by Western blot (right panel). We found that multiple myeloma cells depleted of p100 were significantly more resistant to treatment with GSK3 inhibitor (red box), demonstrating that p100 accumulation contributes to the cytotoxicity of GSK3 inhibitors in multiple myeloma. Depletion of p100 was not sufficient to prevent the cytotoxic effect of MLN4924 or bortezomib.

Slide 42: FBXW7 may function as an oncogene or tumor suppressor depending on the genetic background of the cancer

On the basis of the work presented here, we propose an alternate function for FBXW7 that is dependent on the genetic context of the tumor. In the case of T cell leukemias and solid carcinomas, it is clear that FBXW7 has tumor-suppressive properties. Mutation of *Fbxw7* in these cancers impairs substrate binding, leading to the aberrant stabilization of oncoproteins, such as nothc, c-myc, and cyclin E. Our data suggest that in B cell malignancies—like multiple myeloma and probably non-Hodgkins lymphomas, both of which also rely on NF-κB signaling for their survival, similar to multiple myeloma—FBXW7 plays a prosurvival role. The clearance of nuclear p100 through the activity of FBXW7 and GSK3 is necessary to sustain NF-κB signaling and survival of B cell cancers. In this context, we propose that the FBXW7-GSK3-p100 mechanism represents a viable therapeutic target for B cell malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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