Imatinib mesylate (Gleevec) inhibits Notch and c-Myc signaling: Five-day treatment permanently rescues mammary development

Robert Callahan, Barry A Chestnut and Ahmed Raafat

Basic Research Laboratory, National Cancer Institute, National Institutes of Health, Bethesda, MD 20814, USA

Corresponding author: Ahmed Raafat. Email: raafata@mail.nih.gov

Abstract

Wap-Int3 transgenic females expressing the *Notch4* intracellular domain (designated Int3) from the whey acidic protein promoter exhibit two phenotypes in the mammary gland: blockage of lobuloalveolar development and lactation, and tumor development with 100% penetrance. Previously, we have shown that treatment of Wap-Int3 tumor bearing mice with Imatinib mesylate (Gleevec) is associated with complete regression of the tumor. In the present study, we show that treatment of Wap-Int3 mice during day 1 through day 6 of pregnancy with Gleevec leads to the restoration of their lobuloalveolar development and ability to lactate in subsequent pregnancies in absence of Gleevec treatment. In addition, these mice do not develop mammary tumors. We investigated the mechanism for Gleevec regulation of Notch signaling and found that Gleevec treatment results in a loss of Int3 protein but not of Int3 mRNA in HC11 mouse mammary epithelial cells expressing Int3. The addition of MG-132, a proteasome inhibitor, shows increased ubiquitination of Int3 in the presence of Gleevec. Thus, Gleevec affects the stability of Int3 by promoting the degradation of Int3 via E3 ubiquitin ligases targeting it for the proteasome degradation. Gleevec is a tyrosine kinase inhibitor that acts on c-Kit and PDGFR. Therefore, we investigated the downstream substrate kinase GSK3 β to ascertain the possible role that this kinase might play in the stability of Int3. Data show that Gleevec degradation of Int3 is GSK3 β dependent. We have expanded our study of the effects Gleevec has on tumorigenesis of other oncogenes. We have found that anchorage-independent growth of HC11-c-Myc cells as well as tumor growth in nude mice is inhibited by Gleevec treatment. As with Int3, Gleevec treatment appears to destabilize the c-Myc protein but not mRNA. These results indicate that Gleevec could be a potential therapeutic drug for patients bearing *Notch4* and/or c-Myc positive breast carcinomas.

Keywords: Mammary gland development, cancer, Notch, c-Myc, Gleevec, ubiquitin, proteasome

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Introduction

The Notch signaling pathway is involved in cell fate decisions of tissues and organs in several different organisms.¹ In mammals, the Notch transmembrane receptor gene family comprises four members: *Notch1*, 2, 3, and 4.^{1,2} Upon ligand activation, the intracellular domain (ICD) of Notch receptor is proteolytically cleaved releasing the ICD. In the case of *Notch4* activation, the ICD is termed Int3. Inappropriate Notch signaling has been implicated in cancer of several tissues in humans and animal model systems.^{1,2}

Three members of the murine *Notch* gene family: *Notch1*, 3, and 4 have been found to be targets of the mouse mammary tumor virus (MMTV), producing truncated genes. The three genes are truncated upstream of the region encoding the transmembrane domain, and the resulting gene products of *Notch1*, 3, and 4 (Int3) encode the transmembrane and ICD of the proteins. The biological consequences

of expression of these truncated genes on mammary gland development and tumorigenesis have been studied in vitro and in vivo.^{1–9} Expression of truncated murine *Notch1* or 3 in the mammary gland under the control of MMTV resulted in impaired lobuloalveolar development, repression of β -casein expression in addition to mammary tumor development.⁸ The HC11 mouse normal mammary epithelial cells are not capable of anchorage-independent growth but have retained the capability to differentiate and express milk proteins in response to lactogenic hormones.¹⁰ Expression of Int3 in these cells conferred the capability for anchorage-independent growth in soft agar.¹¹ Expression of truncated *Notch4* (Int3) under the control of MMTV or whey acidic protein promoter (Wap) produced two phenotypes. In MMTV-Int3 mice, mammary ductal development was blocked at the epithelial rudiment stage. Upon pregnancy, ductal elongation resumed and ducts filled the mammary fat pad,^{3,4} indicating inhibition of Int3

signaling by pregnancy hormones. These mice did not lactate due to the lack of lobuloalveolar development, but they did develop mammary tumors. The Wap-Int3 mice exhibit normal ductal development, but during pregnancy, the lobuloalveolar development is severely impaired and therefore, these mice also lack the ability to lactate.⁵ In addition, 80% of these mice develop mammary tumors by the second pregnancy⁹ and 100% of the nulliparous Wap-Int3 mice develop mammary tumors by 70 weeks.⁵ These two phenotypes observed in the MMTV- and Wap-Int3 mice are a consequence of two different components of Notch signaling. Impairment of normal mammary development is due to the interaction of Int3 with the transcription repressor/activator Rbpj. While the other component of Notch signaling that is associated with mammary tumor development is independent of Int3 interaction with Rbpj.¹²

It has been shown that Imatinib mesylate (Gleevec) interferes with *Notch4* signaling.^{13–15} Previously, we have shown that treatment of Wap-Int3 tumor bearing female mice with Gleevec is associated with complete regression of mammary tumors.¹³ Regulation of Notch signaling is complex.¹⁶ Phosphorylation of Notch has been indirectly correlated with Notch activation and nuclear translocation.¹⁷ Notch-ICD is phosphorylated by multiple kinases at several residues. Phosphorylation of Notch-ICD by glycogen synthase kinase 3 β (GSK3 β) occurs at the C-terminal end to the ANK repeats and has different effects on Notch signaling.¹⁸ For example, it inhibits hairy and enhancer of split 1 (*Hes1*) activation by *Notch2*¹⁷ but stabilizes *Notch1*-ICD by increasing its half-life.¹⁹ GSK3 β is a ubiquitously expressed conserved serine/threonine kinase. It has a complex regulation that involves phosphorylation/inhibition and dephosphorylation/activation and as a result differential recognition of target substrates.²⁰ GSK3 β activity is regulated through the canonical Wnt signaling pathway,²¹ also it is modulated by the phosphatidylinositol 3 kinase/Akt (PI3K/Akt).²² It has been shown that PI3K activity is regulated by stem cell factor (SCF)/c-Kit and platelet-derived growth factor receptor (PDGFR),^{23,24} which are regulated by *Notch4*/Int3,¹³ linking *Notch4*/Int3 to GSK3 β .

In eukaryotic cells, protein degradation occurs in the lysosomes, but the main mechanism of protein degradation is the ubiquitin-proteasome system.^{25,26} The ubiquitin pathway is a conserved and critical pathway for regulation of many cellular processes such as protein turnover, trafficking, and transcription. The basic pathway depends on marking the target protein with ubiquitin, a 76-amino acid peptide. This is done through the action of three enzymes, the ubiquitin-activating enzyme (E1) that transfers ubiquitin, to a ubiquitin-conjugating enzyme (E2) and E3 ubiquitin ligase, which combines with an E2 protein to transfer ubiquitin to the target protein. Notch receptors and their ligands are potential substrates for ubiquitin addition by E3 ligases.^{27–29} It has been demonstrated that Notch-ICD is rapidly polyubiquitinated and degraded through the proteasomal pathway, and the E3 ubiquitin ligase accounting for these modifications is Fbw7/Sel-10.^{30–32}

To elucidate the mechanism by which Gleevec inhibits Notch signaling, we established in vitro and in vivo systems using cell lines and transgenic mice. We found that Gleevec

regulates Int3 in post-transcription fashion, where Int3 protein expression decreases in the presence of Gleevec without a change in mRNA levels. In the presence of proteasome inhibitor (MG-132), Gleevec increases ubiquitination of Int3 in HC11-Int3 cells. Also, Gleevec decreases the phosphorylation level of GSK3 β . The activation of GSK3 β reduces Int3 expression levels in HC11-Int3 cells, and significantly reduces the ability of these cells for anchorage-independent growth in soft agar in presence of Gleevec. This data indicate that Gleevec inhibits Notch signaling by increasing proteasomal degradation of the Notch ICD. As with Int3, Gleevec treatment had the same effects on c-Myc tumors. Thus, Gleevec could be a potential therapeutic drug in Notch and/or c-Myc positive breast cancers.

Materials and methods

Mice and experiment design

FVB/N and Wap-Int3 female mice used in this study are maintained in our colony and have been described previously.^{5,33} To treat pregnant mice with Gleevec, 10-week-old females were bred overnight with males, then the males were removed. The plugged females were treated with Gleevec using Alzet miniosmotic pumps (Model 2001, pumping rate 1 μ l/h, Durect Corp., Cupertino, CA, USA), implanted subcutaneously on the dorsal side of the plugged mice. They were used to deliver a subcutaneous dose of Gleevec (21 mg/mouse/week) or saline (control) for five days (day 1 to day 6 of first or second pregnancy only). It is during this period of pregnancy that secretory lobuloalveolar units develop in the mammary gland. Since, not all plugged females become pregnant, non-pregnant females were excluded from the experiment. Gleevec was generously provided by Novartis Pharma (Basel, Switzerland).

The athymic nude female mice are purchased from NCI-Frederick Mouse Repository. Mice were kept under standard laboratory conditions according to the guidelines of the National Cancer Institute. This study was approved by the Institutional Ethics Committee for Laboratory Animals used in Experimental Research. In this study, we have used the HC11 mouse normal mammary epithelial cell line as control cell line and HC11-Int3 and HC11-c-Myc as experimental cell lines.

Mammary morphology, histology, and epithelial content analysis

Representative mammary tissue specimens were collected from FVB/N and Wap-Int3 female mice at indicated times and developmental stages. Mammary whole mounts were prepared from the fourth abdominal gland, as previously described.³⁴ Briefly, mammary glands were fixed for 2 h in freshly prepared 4% paraformaldehyde prepared in phosphate-buffered saline (pH 7.4), rinsed through several changes of phosphate-buffered saline buffer, dehydrated in ethanol, stained overnight with carmine alum, and examined grossly under a dissecting microscope, or embedded in paraffin blocks. For histology analysis, paraffin sections (5 μ m thick) were placed on slides, deparaffinized, and stained with hematoxylin and eosin. Epithelial content

quantification was performed using whole mounts and an AxioVision digital image processing software analysis system (Zeiss, Oberkochen, Germany). The quantification is based on surface area of the whole mount occupied by the mammary epithelial structures. This technique is well known and has been used in numerous studies.^{33,34}

Transplantation of HC11-derived mammary epithelial cells into mammary glands

Cells were grown and mixed with Matrigel as previously described.³⁵ Ten-week-old nulliparous athymic nude female recipient mice were not bred and were palpitated twice a week. HC11-Int3¹¹ and c-Myc (Addgene, Cambridge, MA) transfected HC-11 cells were grown to 70% confluence, harvested, and counted. One million cells of the indicated cell lines were mixed with 25% Matrigel in 1:1 ratio and injected in the left and right #4 inguinal mammary gland of 10-week-old nulliparous nude mice. Tumor weight was determined as described previously.¹³ Tumors were allowed to grow until they reached 400 mg, at which point, the tumor-bearing mice were treated with Gleevec. Alzet miniosmotic continuous release pumps (Model 2001, pumping rate 1 μ l/h, Durect Corp., Cupertino, CA, USA) were implanted subcutaneously on the dorsal side of the mouse. The Alzet pumps were used to deliver a subcutaneous dose of Gleevec (21 mg/mouse/week)¹³ or saline (control). Mice were kept under standard laboratory conditions according to the guidelines of the National Cancer Institute.

Quantitative reverse transcriptase polymerase chain reaction

Quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) was conducted using RNA from control HC11 and experimental HC11-Int3 or HC11-c-Myc cell lines. Cells were pretreated with Gleevec (2.5 μ M) for 48 h prior to RNA isolation. The RNA was obtained by using the RNeasy isolation kit (Qiagen, Valencia, CA) and quantified using the NanoDrop (Thermo Scientific, Waltham, MA). Exactly, 150 ng of RNA was used in the Agilent qRT-PCR kit and amplified in the Mx3000P System (Agilent Technologies, Santa Clara, CA), according to the manufacturer's instructions. Analysis was done using the Mx-Pro qPCR software (Agilent, Santa Clara, CA) with HC11 wild-type cells as the calibrator control and GAPDH as the normalizing control for all samples tested. The following primers were used for qRT-PCR: c-Myc primers, Forward: 5'-GATCAGCTCTC CTGAAAAGAGC-3', Reverse: 5'-CGCAGATGAAATAGGGCTGTAC-3', Int3 primers, Forward 5'-CGATGTGAGAAAG ACATG-3', Reverse 5'-GCTCCGGGGAGATC AAGG-3'; GAPDH primers, Forward 5'-AAGGTCATCCAGAGCTGAA-3', Reverse 5'-CTGCTTACCACC TTCTTGA-3'.

MG-132 treatment, GSK3 β inhibitor, Gleevec treatment and siRNA gene silencing

To inhibit proteasomal degradation, cells were grown and treated with the proteasome inhibitor MG-132 for 4 h at 20 μ M. To inhibit GSK3 β , cells were grown and treated

with the GSK3 β inhibitor SB216763 (10 μ M) or the inhibitor 6-bromindirubin-3'-oxime (BIO-6; 0.2 μ M) to inhibit endogenous GSK3 β in HC11 and HC11-Int3 cells in the presence or absence of Gleevec (2.5 μ M). Gene silencing using siRNA was done as previously described.¹³ The cells subjected to Gleevec were treated for 48 h. Knockdown of proteins was achieved by siRNA (siGenome SMART pool, Dharmacon/Thermo Scientific) according to the manufactures protocol. The siRNA transfection transfections were done with a final concentration of 50 nM in six-well plate or scaled up 10 cm-plate formats. Whole-cell protein lysates were harvested for Western blot analysis or cells were used in anchorage-independent soft agar assays.

Western blot and immunoprecipitation of cultured cells

Cells were harvested in radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA) supplemented with the protease inhibitor cocktail set 1 (Calbiochem, San Diego, CA). Total protein concentration was determined with the BCA Protein Assay Kit (Pierce Biotechnology Rockford, IL). Protein lysates (50 μ g total) were resolved on gradient (4–20%) Tris-glycine gels (Invitrogen, Grand Island, New York) and transferred to nitrocellulose filter membranes by electroblotting. The nitrocellulose membranes were blocked with 5% bovine serum albumin in Tris-buffered saline (TBS) supplemented with 0.1% tween (TBS-T). After overnight incubation with proper primary antibodies, anti-Notch4 (Millipore, Temecula, CA 07-189), anti-ubiquitin (Santa Cruz Biotechnology, Dallas, TX, sc-8017), anti-phospho-GSK3 β (Cell Signaling Technology Beverly, MA, D3A4-9322S), anti-CDK8 (Cell Signaling Technology, G398-4101), anti-integrin-linked kinase (ILK) (Cell Signaling Technology, 4G9-3856), anti-GSK3 β (Cell Signaling Technology, 27C10-9315), anti-c-Myc (Cell Signaling Technology, 9402), anti-GAPDH (Sigma, St. Louis, MO, G8795), and anti-tubulin (Santa Cruz Biotechnology, sc-5286), the blots were rinsed and incubated with the proper peroxidase-conjugated secondary antibody. Proteins were visualized using the ECL Western blotting detection system (Amersham, GE Healthcare Pittsburgh, PA).

For immunoprecipitation, cell lysates were harvested from Gleevec or Gleevec and MG-132-treated HC11 and HC11-Int3 cells as described above. A total of 500 μ g of total protein lysate was immunoprecipitated using 1 μ g of the Notch4 antibody (Millipore, 07-189). The immune complexes were captured using protein G agarose (Thermo Fisher Scientific Grand Island, NY) and run on 4%–20% Tris-glycine gels before being transferred to nitrocellulose filter membrane by electroblotting. The blots were rinsed, blocked, and incubated with anti-ubiquitin (Santa Cruz Biotechnology, sc-8017) and processed as described above.

Soft agar and luciferase assay

Soft agar colony formation assay is a common method used to monitor anchorage-independent growth, which measures proliferation in a semisolid culture media after three

to four 4 weeks. This traditional method has been widely used and published since 1977. Soft agar assay is considered to be a hallmark and one of the most stringent tests for cells malignant transformation.^{11,36} The soft agar assay utilizes an agar medium to assess the transformability of cells in an anchorage-independent manner. The agar is a 2 × RPMI medium comprises Gibco RPMI medium 1640 powder, dissolved in 450 ml Dep-treated H₂O, 2 g of sodium bicarbonate, 100 ml FBS, and 11 ml Pen/strep. The agar consists of a 2-ml bottom layer (1.2% agarose in H₂O with 2 × RPMI) and a 2-ml top layer (0.5% agarose in H₂O with 2 × RPMI). The cells are harvested and counted for suspension (15,000 cells/ml) in the top layer of agarose in the presence or absence of the desired concentration of Gleevec and/or GSK3 β inhibitor. The cell suspension in agarose is allowed to solidify before adding an aqueous layer of HC11 complete medium (500 ml) to non-treated wells or HC11 complete medium with desired concentration of Gleevec and/or GSK3 β inhibitor to treated wells. For the luciferase assay, cells were grown in Dulbecco's Modified Eagle's medium medium containing 10% fetal bovine serum. Transfection and luciferase assays were conducted as previously described.³⁵

Statistical analysis

All quantitative data are expressed as mean $\pm$ standard deviation or standard error of the mean. Statistical analysis among groups was performed with one-way analysis of variance. All values with a *P* value of *P* < 0.05 was considered statistically different.

Results

Inhibition of Notch signaling during early pregnancy induces mammary lobuloalveolar development and lactation

We have previously shown that expression of Int3 from either the MMTV long terminal repeat promoter or Wap in transgenic female mice blocks normal mammary lobuloalveolar development and the ability of these females to lactate.³⁻⁵ In addition, 100% of the Wap-Int3 nulliparous females develop mammary adenocarcinomas by 70 weeks⁵ of age and 80% develop mammary tumors by the second pregnancy.⁹ To investigate the consequences of blocking of Int3 signaling on mammary lobuloalveolar development, Wap-Int3 female mice received a treatment of 21 mg/week of Gleevec 24 h after mating and a vaginal plug was observed. Treatment was continued until day 6 of first pregnancy only as described in the "Materials and methods" section. Gleevec-treated Wap-Int3 females were able to lactate and nurse their pups in the first pregnancy and all subsequent pregnancies in absence of Gleevec treatment. Morphological (Figure 1(a)) and histological (Figure 1(b)) analysis of mammary glands showed a significant increase in alveolar development in the Gleevec-treated Wap-Int3 mice compared to the untreated Wap-Int3 female mice. The untreated Wap-Int3 females have had a significantly lower epithelial content than the matching FVB/N females. However, there was no significant

difference between the late pregnant Gleevec-treated Wap-Int3 females when compared to matching pregnant FVB/N females (Figure 1(c)). Thus, the lobuloalveolar development and lactation defect was rescued in the Gleevec-treated Wap-Int3 female mice (Figure 1(a) and (b); Table 1).

Rescue of the Wap-Int3 lactation phenotype after the first pregnancy five-day Gleevec treatment, raised the possibility that Int3 expression is either temporarily blocked during Gleevec treatment or permanently blocked during and after Gleevec treatment. To investigate this hypothesis, mammary tissue was collected from pregnant FVB/N and Wap-Int3 female mice before, during, and after Gleevec treatment. Western blot analyses (Figure 1(d)) showed a reduction in Wap-Int3 expression during Gleevec treatment (Figure 1(d), lanes 3 vs. 2). However, Wap-Int3 expression was not affected after withdrawal of Gleevec treatment (Figure 1(d), lanes 4 vs. 2). In fact, Int3 expression after withdrawal of Gleevec treatment was higher than in the untreated Wap-Int3 mice (Figure 1(d) lanes 4 vs. 2). The increase in Int3 expression is due to the increased number of cells expressing the Wap-Int3 transgene after Gleevec treatment. This is consistent with mammary cells differentiation and induction of alveolar development. FVB/N mice did not show a detectable expression of Int3 by Western blot (Figure 1(d), lane 1). We have shown previously that *Notch4* is the least expressed among the four Notch receptors during normal mammary gland development.³⁷

Surprisingly, Gleevec-treated Wap-Int3 mice were able to lactate in the subsequent pregnancies in the absence of Gleevec treatment (Table 1). To confirm these results, we exposed Wap-Int3 females to the same Gleevec treatment in the second pregnancy. These females were not able to lactate in the first pregnancy (before Gleevec treatment), however, upon exposure to Gleevec in the second pregnancy they were able to lactate, and continued to lactate in the all subsequent pregnancies similar to the first pregnancy-treated Wap-Int3 females. Mammary lobules and alveoli start to expand at day 5 of pregnancy³⁸ and continue their growth and differentiation during gestation to form functionally active glandular structures that produce and secrete milk proteins during lactation. The defect in lobuloalveolar development in the mammary glands of Wap-Int3 mice results in failure to produce milk. Therefore, Gleevec treatment in the first week of gestation blocked Int3 signaling allowing the alveolar progenitor cells to differentiate and give rise to the glandular structures. Also, these results indicate that once the alveolar progenitor cells escape *Notch4* premature termination of the differentiation program, they will not respond to Notch signaling in the subsequent pregnancies. None of the treated mice subsequently developed mammary tumors (Table 1). We have shown that mammary development is *Rbpj* dependent.¹² Also, we have shown that *Rbpj* expression is highest during early pregnancy.³⁷ Therefore, these findings establish a role for Notch signaling as a negative regulator of alveolar development and suggest that Notch signaling is required for the prevention of lactation prior to parturition.

Figure 1 Mammary gland morphology and histology analysis of late-pregnant FVB, Wap-Int3, and Gleevec-treated Wap-Int3 females. Ten-week-old pregnant FVB and Wap-Int3 females were treated with Gleevec or placebo for five days starting at day 1 of pregnancy as describes in the "Materials and Methods" section. Mammary tissue was collected at day 15 of pregnancy. (a) Morphology analysis of mammary glands from late pregnant FVB/N, Wap-Int3, and Gleevec-treated Wap-Int3 mice. Mammary glands of Gleevec-treated Wap-Int3 females are more developed than the Wap-Int3 untreated mice (scale bars: 1.0 mm). (b) Histological analysis of mammary glands from late-pregnant FVB/N, Wap-Int3, and Gleevec-treated Wap-Int3 mice. Analysis show phenotype rescue of alveolar development after Gleevec treatment (scale bars: 20 μ m). (c) Quantification of epithelial content of mammary glands. FVB/N and Gleevec-treated Wap-Int3 mammary glands had significantly higher epithelial content than the untreated Wap-Int3 females ($P < 0.05$ vs. FVB/N). A total of at least five to six mice were used for each experiment. (d) Int3 expression in Wap-Int3 mice mammary glands before, during, and after Gleevec treatment. Wap-Int3 females were treated with Gleevec (lanes 3 and 4) or placebo (lane 2) as described in the "Materials and Methods" section. Mammary tissue was collected at day 3 of the treatment (lane 3), or four days after treatment withdrawal (lane 4). Mammary protein extracts from pregnancy matching FVB/N females was used as a control (lane 1). Endogenous mammary gland Int3 expression is not detectable by Western analysis in FVB mice mammary glands (lane 1), expression level is high in the untreated Wap-Int3 (lane 2), lower in Wap-Int3 during Gleevec treatment (lane 3), and highest after the withdrawal of Gleevec treatment (lane 4). Tubulin was used as a control for equal loading. Analysis has been repeated three times using protein extracts from different mice. (e) Respective measurements of band intensity in lanes 1, 2, 3, and 4 of (d) presented in arbitrary units. Int3 expression is significantly reduced ($P \leq 0.05$) in presence of Gleevec treatment. Each bar represents the mean $\pm$ SEM

Gleevec regulates Int3 expression and signaling through ubiquitin ligase complex

To examine the mechanisms underlying the effect of Gleevec on Int3 signaling, we investigated the effects of Gleevec treatment on Int3 expression in Gleevec-treated HC11 and HC11-Int3 cells. QRT-PCR analysis showed that Int3-mRNA levels

in the Gleevec-treated HC11-Int3 cells are not significantly different from the HC11-Int3 untreated cells (Figure 2(a)). However, Western blot analysis of Int3 expression showed a significant reduction in Int3 protein levels in Gleevec-treated HC11-Int3 cells than the untreated HC11-Int3 cells (Figure 2(b) compare lanes 3 and 4). Densitometry analysis

Table 1 Inhibition of Int3 signaling during early pregnancy induces mammary alveolar development and permanently rescues the phenotype^a.

Strain	Treatment	Dose (mg/week)	Mice no.	Lactation	Tumor
Wap-Int3	None	None	10	No	Yes
FVB/N	Once 1st preg	21	10	Yes	No
Wap-Int3	Once 1st preg	21	10	Yes	No
Wap-Int3	Once 2nd preg	21	10	Yes (after 2nd preg)	Regressed

^aPost-pubertal 10-week-old FVB/N and Wap-Int3 females were treated with saline (0.00) or Gleevec (21 mg/week) for five days. Treatment started at first day of pregnancy and continued until day 6. Gleevec-treated Wap-Int3 females were able to lactate after Gleevec treatment and continued to lactate in the subsequent lactations in absence of Gleevec treatment.

Figure 2 Gleevec regulates Int3 protein levels and signal through the ubiquitin ligase complex pathway. (a) Gleevec effect on Int3-mRNA levels. HC11 and HC11-Int3 cell lines were grown and treated with Gleevec. Total RNA was extracted and used in qRT-PCR analysis as described in the “Materials and Methods” section. No significant difference is observed in Int3-mRNA levels in Gleevec-treated HC11-Int3 cells compared to HC11-Int3 untreated cells (control). Each bar represents the relative quantity of Int3. The experiment was repeated three times independently and qRT-PCR results were normalized against GAPDH-mRNA levels. Results presented as bars representing mean $\pm$ SEM. (b) Gleevec effect on Int3 expression. Western blot analysis of Int3 in HC11 (lanes 1 and 2) and HC11-Int3 (lanes 3 and 4); cell lines were grown in the absence (lanes 1 and 3) or presence of Gleevec (lanes 2 and 4) as described in the “Materials and Methods” section. Int3 is not expressed in the presence of Gleevec. The experiment was independently repeated three times. (c) Respective measurements of band intensity in lanes 3 (HC11-Int3) and 4 (HC11-Int3 + Gleevec) of (b) presented in arbitrary units. Int3 expression is significantly reduced ($P \leq 0.05$) in presence of Gleevec. Each bar represents the mean $\pm$ SEM. (d) To investigate the consequence of Gleevec down-regulation of Int3 expression on Int3 signaling, HC11 and HC11-Int3 cells were transfected with a fixed concentration of luciferase reporter construct under the control of the Hes-1 promoter (0.5 μ g) in the presence or absence of increasing concentrations of Gleevec (0, 0.5, 1.0, 1.5, or 2 μ M), as described in the “Materials and Methods” section. Data show a significant dose-dependent down regulation of Int3 signaling as Gleevec concentration increases. Each bar represents the mean $\pm$ SEM of a minimum of a duplicate of three-independent experiments for each experimental group. $*P \leq 0.05$ compared to HC11-Hes-Luc and $**P \leq 0.05$ compared to HC11-Int3-Hes-Luc. Each bar represents the mean $\pm$ SEM. (e) To dissect molecular mechanism by which Gleevec down-regulate Int3 expression, HC11 (lanes 1 and 2) and HC11-Int3 (lanes 3 and 4) were treated with 0.25 μ M Gleevec for 48 h (lanes 1 and 3) or with 0.25 μ M Gleevec for 48 h and 20 μ M MG-132 proteasome inhibitor for 5 h (lanes 2 and 4) as described in the “Materials and Methods” section. Immunoprecipitation (IP) of protein extracts with Int3 antibody and immunoblotting (wb) with ubiquitin antibody showed accumulation of ubiquitinated Int3 in presence of MG-132. This data point to an increase in Int3 ubiquitination by Gleevec treatment

showed $1.78 \times$ average decrease and significant reduction in Int3 expression in presence of Gleevec (Figure 2(c)). Thus, HC11-Int3 protein expression decreases in the presence of Gleevec without a change in mRNA levels, indicating post-transcription regulation. To further investigate these results, we conducted a dose-dependent down regulation study of

Int3 signaling in presence and absence of Gleevec. HC11 and HC11-Int3 cells were transfected with Hes-1-luciferase reporter plasmid and grown in absence and presence of increasing concentrations of Gleevec. Hes-1 is one of the best characterized Notch-target genes. Hes-1 expression is directly up-regulated by a Notch ICD-RBPJ interaction and

is considered the primary effector of RBPJ-dependent Notch signaling.¹ Hes-1 reporter activity decreased significantly as Gleevec concentration increased (Figure 2(d)). This decrease in Int3 signaling could be a result of Int3 protein reduction in presence of Gleevec.

To further dissect the mechanism by which Gleevec down regulates Int3 protein expression and signaling, we treated HC11 and HC11-Int3 cells with Gleevec in presence and absence of the proteasome inhibitor (MG-132) as described in "Materials and Methods" section. Dose-dependent inhibition of proteasome protein degradation was observed in HC11 cells treated with 20 μ M MG-132 for 4 h (data not shown). MG-132 selectively inhibits the 26S proteasome,³⁹ resulting in accumulation of ubiquitin-conjugated proteins. Degradation of the ubiquitin-conjugated Int3 is reduced in presence of MG-132 (Figure 2(e), compare lanes 3 and 4), suggesting that Gleevec treatment enhances the ubiquitination and subsequent proteasomal degradation of Int3. The light smear seen in lanes 1 and 3 in absence of MG-132 is very typical of proteins that are ubiquitinated.³²

Gleevec activation of GSK3 β and the consequence of this activation on Int3 expression

Glycogen synthase kinase-3 β (GSK3 β)-mediated phosphorylation has been implicated in regulating the proteolytic degradation of potential oncogenic proteins including c-Myc, c-Jun, Cyclin E, and Notch.⁴⁰ Down regulation of Int3 protein expression by Gleevec raised the question of the status of GSK3 β , as an upstream effector of Notch signaling. To address this question, we next chose to examine the status of GSK3 β in presence and absence of Gleevec. Gleevec treatment reduced GSK3 β phosphorylation resulting in GSK3 β activation in HC11 (Figure 3(a) compare lanes 1 and 2) and HC11-Int3 (Figure 3(a), compare lanes 3 and 4). We next quantified the reduction in GSK3 β phosphorylation in Gleevec-treated HC11-Int3 cells. Densitometry analysis of the Western blot showed a significant reduction (1.66 $\times$ average reduction) of GSK3 β phosphorylation in Gleevec-treated HC11-Int3 cells (Figure 3(b)). CDK8 is a kinase that is recruited by Mastermind (MAML1) to phosphorylate Notch, which facilitates Notch proteasomal degradation.⁴¹ Also, ILK binds and phosphorylates *Notch1*-ICD, facilitating proteasomal protein degradation through Fbw7.⁴² To investigate if CDK8 and/or ILK are involved in Int3 degradation by Gleevec treatment, we investigated Gleevec's effect on CDK8 and ILK by Western blot analysis. Control cell line HC11 did not express CDK8 or ILK in absence (Figure 3(c) lane 1) or presence (Figure 3(c) lane 2) of Gleevec. CDK8 and ILK are expressed in HC11-Int3 cells (Figure 3(c) lanes 3 and 4). However, Gleevec treatment did not affect CDK8 or ILK levels (Figure 3(c) lanes 4 vs. 3). This data suggest that CDK8 and ILK do not play a role in Int3 degradation by Gleevec and that this degradation is due to GSK3 β activation by Gleevec.

Having established that Gleevec treatment leads to the degradation of Int3 protein, we sought to further investigate the role of GSK3 β on Int3 degradation. To this end, we used SB216763 a selective GSK3 β inhibitor^{43–45} to knock down GSK3 β activity in HC11 (Figure 3(d) lanes 1–3)

and HC11-Int3 cells (Figure 3(d) lanes 4–6). Cells were treated with SB216763 (Figure 3(d) lanes 2 and 5) or with both GSK3 β inhibitor and Gleevec (Figure 3(d) lanes 3 and 6). GSK3 β inhibition protected Int3 expression in presence of Gleevec (Figure 3(d) lane 6). Densitometry analysis (Figure 3(e), lanes 4–6) of Western blots showed no significant difference of Int3 expression in HC11-Int3 cells treated with SB216763 or with both Gleevec and SB216763 when compared with HC11-Int3 cells. This indicates that Gleevec-induced degradation of Int3 is GSK3 β dependent. The equal band intensity in lanes 4 to 6 points to a lack of Int3 accumulation in the absence of GSK3 β and suggests increase in Int3 half-life.⁴⁶

GSK3 β inhibition rescues Int3 transformation ability in presence of Gleevec

To investigate whether GSK3 β inhibition retrieves Int3 function or not, we investigated the effects of the GSK3 α and β inhibitor BIO-6 on HC11 and HC11-Int3 cells in a soft agar colony formation assay in presence and absence of Gleevec (Figure 4(a)). When we treated HC11-Int3 cells with Gleevec, colony formation was significantly reduced compared to the untreated HC11-Int3 cells. We have previously shown that Gleevec inhibits Int3 ability to transform HC11 cells to anchorage-independent growth in soft agar.¹³ Addition of BIO-6 did not significantly affect the level of HC11-Int3 colony formation in absence of Gleevec but did rescue the transformation ability of Int3 in presence of Gleevec. Since BIO-6 inhibits both GSK3 α and GSK3 β , we treated HC11-Int3 cells with GSK3 β -siRNA to knock-down GSK3 β RNA expression. Western blot analysis of GSK3 β expression in HC11-Int3 cells is shown in Figure 4(b). Compared with untreated HC11-Int3 cells (Figure 4(b), lane1), GSK3 β expression is virtually undetectable in HC11-Int3 cells treated with the GSK3 β -siRNA (Figure 4(b), lane 2). The effect of GSK3 β -siRNA on Int3 function and transformation ability was validated by soft agar assay (Figure 4(c)) of HC11 and HC11-Int3 cells. As expected, soft agar colony formation by control cell line HC11 was significantly lower than the colony formation of the HC11-Int3 cells. Gleevec treatment significantly reduced colony formation by the HC11-Int3 cells compared to the HC11-Int3 untreated cells, indicating reduction in Int3 transformation ability. Inhibition of GSK3 β expression by GSK3 β -siRNA significantly increased HC11-Int3 colony formation in absence of Gleevec. The significant increase in colony formation by HC11-Int3 cells treated with GSK3 β -siRNA could be due to lack of Int3 degradation caused by an increase in Int3 half-life.^{32,46} Thus, treatment of HC11-Int3 cells with Gleevec in absence of GSK3 β blocks the ability of Gleevec to inhibit HC11-Int3 anchorage-independent growth. These results show that loss of GSK3 β does not only protect Int3 degradation from Gleevec treatment but also it renders Int3 active. In addition, it shows that Int3 degradation by Gleevec treatment is GSK3 β dependent.

To investigate whether GSK3 β inhibition affects Int3 signaling (Figure 4(d)); HC11 and HC11-Int3 cells were transfected with constant concentration of Hes-Luc reporter construct, treated with Gleevec and with increasing

Figure 3 Gleevec activation of GSK3 β and consequences of this activation. (a) Gleevec effect on GSK3 β activation. HC11 (lanes 1 and 2) and HC11-Int3 (lanes 3 and 4), cell lines were grown in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of Gleevec; protein was extracted and used in Western blot analysis as described in the "Materials and Methods" section. Results show reduction of GSK3 β -phosphorylation in Gleevec-treated HC11-Int3 cells. Thus, Gleevec treatment activates GSK3 β . The experiment was repeated three times using independent protein extractions. (b) Respective measurements of band intensity in lanes 3 (HC11-Int3) and 4 (HC11-Int3 + Gleevec) of (a) are presented in arbitrary units. Gleevec treatment significantly reduced GSK3 β phosphorylation (* $P \leq 0.05$), resulting in the activation of GSK3 β . Each bar represents the mean $\pm$ SEM. (c) Specificity of Gleevec effect on GSK3 β in Int3 expressing cells. Western blot analysis of CDK8 and ILK in HC11 (lanes 1 and 2) and HC11-Int3 (lanes 3 and 4) cells, in absence (lanes 1 and 3) and presence (lanes 2 and 4) of Gleevec. Gleevec does not affect CDK8 or ILK in HC11-Int3 cells. Accordingly, CDK8 and ILK do not play a role in Int3 degradation by Gleevec. GAPDH was used as an equal loading control. (d) To investigate GSK3 β role in Int3 degradation by Gleevec treatment, HC11 (lanes 1, 2, and 3) and HC11-Int3 cells (lanes 4, 5, and 6) were grown in absence of treatment (lanes 1 and 4), or presence of the GSK3 inhibitor SB216763 (lanes 2 and 5) or presence of both Gleevec and SB216763 (lanes 3 and 6). Protein was extracted and used in Western blot analysis as described in the "Materials and Methods" section. SB216763 protects the Int3 protein from degradation induced by Gleevec treatment (lanes 6). GAPDH was used as an equal loading control. The experiment was repeated at least three times using independent protein extractions. (e) Respective measurements of band intensity in lanes 4 (HC11-Int3 no treatment), 5 (HC11-Int3 + GSK3 β inhibitor), and 6 (HC11-Int3 + GSK3 β inhibitor + Gleevec) of (d) presented in arbitrary units. The data indicates that Gleevec degradation of Int3 is GSK3 β dependent. Each bar represents the mean $\pm$ SEM

concentrations of GSK3 β -siRNA. Interestingly, treatment of HC11-Int3 cells with increasing concentrations of GSK3 β -siRNA led to a dose-dependent up-regulation of Int3 signaling from the *Hes* promoter. This indicates a role for GSK3 β in the regulation of *Notch4* function and signaling.

Gleevec affects on c-Myc signaling

Since GSK3 β has also been reported to phosphorylate c-Myc, we were interested in determining whether Gleevec could also affect c-Myc function and stability in a manner similar to Int3. As shown in Figure 5(a), Gleevec inhibits anchorage-independent growth of HC11-c-Myc cells in soft agar. To determine whether the loss of soft agar growth was due to loss of c-Myc protein in presence of Gleevec, we grew HC11-c-Myc cells in presence and absence of Gleevec and analyzed the protein extracts with Western blot analysis (Figure 5(b)). Gleevec treatment resulted in reduction of Myc protein (Figure 5(b)), however, similar to Int3, this was not due to a significant loss of c-Myc RNA (Figure 5(c)). To further investigate Gleevec's effect on c-Myc signaling, we transfected HC11 and HC11-c-Myc cells with a c-Myc-luc reporter plasmid and grew them in presence and absence of increasing concentrations of Gleevec (Figure 5(d)). The c-Myc reporter activity significantly decreased as Gleevec concentration increased, indicating a decrease of c-Myc signaling in presence of Gleevec.

To ascertain whether Gleevec has the same effect on c-Myc *in vivo*, c-Myc tumor-bearing mice were treated with 21 mg/week of Gleevec. We have previously shown that treatment of mice bearing Wap-Int3 tumors with 21 mg/week of Gleevec resulted in tumor regression.¹³ Treatment of Wap-Int3 and c-Myc tumor-bearing mice resulted in a significant reduction in mammary tumor weight (Figure 5(e)). Histological analysis of hematoxylin and eosin (H&E)-stained liver and kidney sections from Gleevec-treated mice did not show any evident morphological alterations such as necrosis or inflammatory changes due to toxicity (data not shown). These results demonstrate that c-Myc mammary tumors are sensitive to Gleevec and further provide a strong rationale that the degradation of c-Myc by Gleevec treatment likely contributes to c-Myc tumor regression.

Discussion

To investigate the mechanism by which Gleevec inhibits Int3, we generated several cell lines in which Int3 is expressed in addition to using the Wap-Int3 mice. The Wap-Int3 transgenic mice exhibit two phenotypes with 100% penetrance. One of these is mammary tumor development in all females. The other phenotype is the blockage of mammary gland lobuloalveolar development resulting in an inability of Wap-Int3 females to lactate. Expression of Wap-promoter driven transgene is different from

Figure 4 GSK3 β inhibition rescues Int3 transformation ability in the presence of Gleevec. (a) To validate the effect of GSK3 β inhibition on Int3 transformation ability, HC11 and HC11-Int3 cells were treated with Gleevec and/or GSK3 inhibitor (BIO-6) and tested for their ability to grow in soft agar as described in the "Materials and Methods" section. * $P < 0.05$, Gleevec treated versus untreated; No significant difference between BIO-6 treated and HC11-Int3 untreated or Gleevec plus BIO-6 treated versus HC11-Int3 untreated. The experiment was repeated three times. Each bar represents the mean $\pm$ SEM. (b) To investigate the impact of GSK3 β inhibition on Int3 function, HC11-Int3 cells were grown and treated with GSK3 β -siRNA. Protein was extracted and used in Western blot analysis as described in the "Materials and Methods" section. GSK3 β expression is reduced more in GSK3 β -siRNA-treated cells (lane2) than in the untreated cells (lane 1), indicating the efficiency of GSK3 β -siRNA in blocking GSK3 β expression. (c) In absence of GSK3 β , Int3 maintains its transformation ability in Gleevec-treated cells. HC11 and HC11-Int3 cells were grown and treated with Gleevec and/or GSK3 β -siRNA and tested for their ability to grow in soft agar as described in the "Materials and Methods" section. HC11-Int3 cells did not lose the ability to grow in soft agar in the presence of Gleevec when treated with GSK3 β -siRNA. * $P < 0.05$, Gleevec treated versus untreated; ** $P < 0.05$, GSK3 β -siRNA treated versus HC11-Int3 untreated. The experiment was repeated three times. Each bar represents the mean $\pm$ SEM. (a) and (c) Data show that blocking of GSK3 β expression allowed HC11-Int3 cells to maintain their anchorage-independent growth in presence of Gleevec. (d) To investigate the effect of GSK3 β inhibition on Int3 signaling, HC11 and HC11-Int3 cells were transfected with a fixed concentration of luciferase reporter construct under the control of the Hes-1 promoter (0.5 μ g) in the absence or presence of Gleevec (1.0 μ M), as described in the "Materials and Methods" section. Each bar represents the mean $\pm$ SEM of a minimum of a duplicate of three-independent experiments for each experimental group. * $P \leq 0.05$, compared to HC11, ** $P \leq 0.05$ compared to HC11-Int3-Hes-Luc

Figure 5 Gleeevec effect on c-Myc function and stability. (a) Dose response effect of Gleeevec treatment on anchorage-independent growth of HC11-c-Myc cells. HC11, HC11-Int3, and HC11-c-Myc cell lines were grown and treated with increasing doses of Gleeevec (0.00, 0.625, 1.25, and 2.5 M) and tested for their ability to grow in soft agar as described in the "Materials and Methods" section. Both HC11-Int3 and HC11-c-Myc exhibit decreased anchorage-independent growth in the presence of Gleeevec. HC11-Int3 cells are used as positive control. *** $P \leq 0.05$ compared to matching HC11 treatment, ** $P \leq 0.05$ compared to HC11-Int3 control, and * $P \leq 0.05$ compared to HC11-c-Myc control. (b) Gleeevec's effect on c-Myc expression. Western blot analysis of HC11 (lane 1), HC11-c-Myc (lane 2), and Gleeevec-treated HC11-c-Myc (lane 3) cell lines protein extracts. Cells were grown and protein extracts prepared as described in the "Materials and Methods" section. c-Myc protein expression decreased in the presence of Gleeevec. Tubulin was used as an equal loading control. (c) Quantitative RT-PCR analysis of c-Myc gene expression in HC11, HC11-c-Myc, and HC11-c-Myc cells treated with Gleeevec, cells were grown as described in the "Materials and Methods" section. No significant difference is observed in c-Myc mRNA levels in the presence of Gleeevec. Each bar represents the relative quantity of *Notch4*/Int3. The experiment was repeated three times using independent RNA extractions. QRT-PCR results were normalized against GAPDH mRNA levels. Results presented as bars representing mean $\pm$ SEM. (d) Dose-dependent down regulation of c-Myc signaling as Gleeevec concentration increases. HC11 and HC11-c-Myc cells were transfected with a fixed concentration of c-Myc-luciferase reporter construct and grown in the presence or absence of increasing concentrations of Gleeevec (0, 0.5, 1.0, 1.5, or 2 μ M), as described in the "Materials and Methods" section. Data show a significant dose-dependent down regulation of c-Myc signaling as Gleeevec concentration increases. Each bar represents the mean $\pm$ SEM of a minimum of a duplicate of three-independent experiments for each experimental group. * $P \leq 0.05$ compared to control (HC11 + c-Myc-Luc) and ** $P \leq 0.05$, compared to Gleeevec-treated HC11-c-Myc + c-Myc-Luc. (e) In vivo effects of Gleeevec on HC11-Int3 and HC11-c-Myc tumors. Mice bearing primary Int3 or c-Myc mammary tumors were treated with 21 mg/week Gleeevec for seven days. Gleeevec was administered using subcutaneous osmotic pumps (continuous release) as described in the "Materials and Methods" section. Continuous release of Gleeevec inhibits HC11-Int3 and HC11-c-Myc mammary tumor growth in vivo, resulting in significant tumor regression. Each data point indicates the mean $\pm$ SEM weight from a minimum of five to six different mice measured at the specified day. * $P \leq 0.05$ compared to day 1 HC11-Int3 and ** $P \leq 0.05$, compared to day 1 HC11-c-Myc

expression of the endogenous Wap gene. Expression of the endogenous Wap reaches maximum activity in the mammary gland at late pregnancy and lactation. In contrast, Wap-mRNA is detected in primary mouse estrogen receptor knockout mammary epithelial cells preparations at day 7 of first pregnancy.⁴⁷ In addition, Jhappan et al.⁴⁸ showed that the Wap promoter is minimally active in mammary glands of virgin and seven-day pregnant mice. In 1995, Kordon et al.⁴⁹ showed that the Wap promoter is active in mammary glands of Wap-LACZ mice during estrus. Also, they showed that few cells in the mammary glands of nulliparous mice express Wap protein. This data explain the lack of alveolar development in the Wap-Int3 mice mammary glands,⁵ where expression of Int3 transgene in early pregnancy under the control of the Wap promoter transgene blocks the lobuloalveolar development process.

Int3-induced blockage of mammary gland lobuloalveolar development is dependent on the transcription activator/repressor Rbpj whereas Int3-induced mammary tumorigenesis is independent of Rbpj.¹² In an earlier study, we found that treatment of Wap-Int3 tumor bearing female mice with Gleevec caused the tumors to regress.¹³ In the present study, pregnant Wap-Int3 female mice received a treatment of Gleevec for five days starting at the first day of pregnancy. Gleevec was administered using continuous release pumps. The treated mice developed alveolar structures and were able to lactate. Surprisingly, mammary gland development occurred normally in all subsequent pregnancies in absence of Gleevec treatment. In addition, these mice did not develop mammary tumors, even though Int3 expression was detectable at high levels after withdrawal of Gleevec treatment (Figure 1(d)). In the normal mouse mammary gland, lobules and their alveoli are formed at puberty and remain in a primitive form at puberty and in virgin mice. They start to grow significantly in early pregnancy and continue their growth and differentiation during pregnancy to form an epithelial rich glandular structure with mature alveoli. Bruno et al.⁵⁰ showed that lobuloalveolar development occurred in Wap-Int3-positive mammary cells when mixed with wild-type mammary epithelial cells and inoculated into the epithelium-divested mammary fat pads of immune-compromised females. The interpretation was that the capacity to produce secretory structures was present in Wap-Int3 females mammary glands but was not expressed because cells affected by Int3 did not supply niche signals. Therefore, it is possible that as Int3 is degraded in response to Gleevec treatment, the mammary niche changes to normal, allowing alveolar progenitor cells to differentiate.

Several differences have been reported between mammary glands of the nulliparous and primiparous female mice. In the nulliparous mouse, mammary gland and during first pregnancy, there is a requirement for DNA synthesis to facilitate terminal mammary differentiation, this requirement is not needed in the second pregnancy for the mammary gland to respond to pregnancy hormones and to lactate.^{51,52} It has also been reported that, overexpression of *Notch1* or 3 decrease proliferation of ductal and alveolar cells at puberty and early pregnancy.⁸ Therefore, it is possible that Int3 expression in the mammary gland

during early pregnancy blocks DNA synthesis and as a result prevents differentiation and lobuloalveolar development. In the treated Wap-Int3 female mice, continuous lactation in the subsequent pregnancies in absence of Gleevec treatment could be due to the gland retention of a long-term memory of lactation, where long lasting alterations in the mammary genome took place after the first pregnancy.⁵³ These established genomic alterations make the mammary gland more responsive to pregnancy hormones. The majority of the genomic alterations target specific genes that are up-regulated during pregnancy and also affect sites occupied by Stat5a,⁵³ which is essential for mammary alveolar development.⁵⁴ Thus Gleevec-treated Wap-Int3 female mice mammary glands will be more responsive to pregnancy hormones after Gleevec treatment and the first lactation. This established parous cell population allows mammary glands to lactate in subsequent lactations. In addition, parity-identified mammary epithelial cells (PI-MECs) are present in nulliparous female mice and inducible in vitro. They are lobule-limited progenitor cells.⁵⁵ It is possible that the PI-MECs are targets of Int3, as a result, they do not differentiate in mammary glands of pregnant Wap-Int3 females. Only upon blocking of Int3 signaling, the PI-MECs are able to differentiate and form lobuloalveolar structures and from this point on they do not respond to Int3 signaling.

Ductal, alveolar, and myoepithelial epithelial cells in the mammary gland are generated from stem cells in a coordinated fashion to yield a broad network of branching ducts in the post pubertal mammary gland and secretory alveolar units during pregnancy. The luminal lineage comprises both ductal and alveolar epithelial cells that line the ducts and constitute the alveolar units that expand during pregnancy.⁵⁶ In normal human mammary cells, Notch signaling acts on both stem cells and progenitor cells, affecting their proliferation and lineage specific-differentiation.⁵⁷ Targeted disruption of Notch signaling in the mouse mammary gland during pregnancy has revealed that during pregnancy Rbpj-dependent Notch signaling regulates lobuloalveolar development by maintaining of luminal cell fate and prevention of uncontrolled basal cell proliferation.⁵⁸ In mammary epithelial cells, phenotypic response to Notch signaling is dependent on Notch signaling dose activation.⁵⁹ Thus, the phenotype rescue in the pregnant Wap-Int3 females and their ability to lactate after Gleevec treatment suggest that the mammary gland phenotype in up-regulated^{3,4,5,8,9} and down-regulated^{12,13} Notch signaling models is based on the degree and timing of up or down-regulation of Notch signaling.

Shown in Figure 6 is a model in which we speculate that in the normal mouse mammary gland, during pregnancy mammary progenitor cells (designated Type A) responsive to Notch/*Rbpj* signaling and expressing endogenous Notch, give rise to two different types of cells (designated Types B and D). Type D cells have the ability to proliferate in response to Notch/*Rbpj*-independent signaling. Type B cells are alveolar committed cells that do not express endogenous Notch, but they are responsive to Notch/*Rbpj*-dependent signaling. Expression of Wap-Int3 in Type B cells prevents them from progressing to Type C alveolar

Figure 6 Proposed model for the effect of Int3 signaling on normal mammary gland development. In the normal mouse mammary gland, during pregnancy mammary progenitor cells responsive to Notch/Rbpj signaling and expressing endogenous Notch (a), give rise to two different types of cells. The first type are cells responsive to Notch/Rbpj-independent signaling (d), these cells have the ability to proliferate in response to Notch signaling. Second type is the alveolar committed cells (b), these cells do not express endogenous Notch but they are responsive to Notch/Rbpj signaling. The (b) cells give rise to (d) cells, also they give rise to the alveolar progenitor cells (c) that are not responsive to Notch signaling and differentiate producing the alveolar structures. Overexpression of Int3 induces the proliferation of (d) cells resulting in mammary tumor development and inhibits (b) cells production, blocking alveolar development. Gleevec treatment leads to the degradation of Int3, as a result tumor development is significantly decreased and Notch blockage of alveolar cells development is released

progenitor cells that are not responsive to Notch signaling but can differentiate to produce the alveolar structures that are productive during lactation. We speculate that Gleevec treatment leads to the degradation of Int3 signaling permitting the progression of Type B cells to Type C progenitor cells that are unresponsive Int3 *Rbpj*-dependent and *Rbpj*-independent signaling. Consistent with this conclusion is the observation that after Gleevec treatment has been removed, Int3 protein can be detected in the mammary gland during subsequent pregnancies and the mice lactate and do not develop mammary tumors.

Several transgenic models and hormone receptor mutant mouse strains studies revealed that activation of specific signals is required for maintaining the integrity, function, or development of mammary alveoli. For example, epithelial ER α signaling is required for ductal elongation and, directly or indirectly for subsequent side branching and alveologenesis.^{60,61} Progesterone receptor⁶²; the transcription factor Gata3,⁶³ prolactin,⁶⁴ and the transcription factor ELF5⁶⁵ knockout mice did not lactate due to a lack or reduction of lobuloalveolar differentiation/development. In the young adult mouse mammary gland, progesterone regulates side branching, while in pregnancy, the steroid hormones estrogen and progesterone, in addition to prolactin all play roles in alveolar expansion.⁶⁶ Interestingly, Notch signaling directly or indirectly interacts with or cross talks with estrogen,⁶⁷ progesterone, progesterone receptor,⁶⁸ Gata3,^{69,70} prolactin, and the Elf5 transcription factor.⁷¹

Previously, we found that c-Kit expression is up-regulated in Wap-Int3 mammary tumors. Gleevec specifically inhibits c-Kit and platelet-derived growth factor receptor- α (PDGFR α) in mammary glands of Wap-Int3 mice.¹³ Here, we attempted to further define the mechanism

by which Gleevec blocks Int3 signaling and found that it causes the destabilization of the Int3 protein leading to its ubiquitination and subsequent degradation by the proteasomes. Ubiquitin is a polypeptide of 76 amino acids, and it can be conjugated to a substrate. Ubiquitination leads to conformational changes in the substrate and as a result targets it to several fates among them proteasomal degradation.⁷² Ubiquitination is catalyzed by three enzymes: E1 Ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. E3 ubiquitin ligases are the only enzymes in the ubiquitination process to be specific for the substrate.^{29,73} Notch-ICD contains a canonical PEST sequence, which is associated with short lifetime molecules²⁹ since it is associated with phosphorylation and ubiquitination.⁷⁴ The present study identifies a novel mechanism for Int3 regulation of mammary gland development through the direct effects of GSK3 β . A primary effect of Gleevec treatment is the inhibition of GSK3 β phosphorylation, unphosphorylated GSK3 β is activated, and phosphorylates Int3 marking it for ubiquitination. The Notch-ICD is ubiquitinated by E3 ubiquitin ligases,²⁸ such as Fbw7 (also known as Cdc4 and Sel10), that can ubiquitinate Notch-ICD within its PEST domain, leading to its proteasomal degradation.^{30–32,39} It has also been shown that the interaction between Sel-10 and Notch proteins is phosphorylation dependent,³² suggesting that Notch-ICD phosphorylation precedes its ubiquitination. Not much is known about kinases that phosphorylate the c-terminus and regulate Notch, but we can speculate that these kinases have a negative regulatory function on Notch signaling.

Activation of c-Kit and PDGFR α expression by Int3¹³ can lead to activation of the phosphoinositide 3-kinase (PI3K) that leads to the phosphorylation of ILK and AKT; in turn,

active ILK and AKT prevent GSK3 β phosphorylation rendering it inactive.²³ This reduces Int3 proteasomal degradation due to lack of phosphorylation by GSK3 β . In presence of Gleevec, phosphorylation of c-Kit and PDGFR is inhibited, leading to GSK3 β activation and Int3 phosphorylation making Int3 a target for ubiquitination and proteasomal degradation. Therefore, blocking of GSK3 β expression by siRNA, SB216763, or BIO-6, rescued Int3 from ubiquitination and subsequent degradation by the proteasome.

These results led us to question whether there are other oncogenes whose cellular transforming activity is inhibited by the action of Gleevec. The c-Myc protein, regulates the expression of several thousand genes involved predominantly in cell growth, proliferation, metabolism, DNA replication, and cytoplasmic tubulin differentiation.⁷⁵ c-Myc overexpression and deregulation is reported in several types of human and animal cancers.⁷⁵ Phosphorylation by GSK3 β affects the stability of several proteins, including c-Myc.^{75,76} Interestingly, c-Myc and Int3 each have a PEST region which when phosphorylated becomes a target for ubiquitination by an E3 ligase.^{29,76,77} Also, Fbw7 is involved in the degradation of c-Myc.^{40,73} In addition multiple ubiquitin ligases have been demonstrated to ubiquitinate c-Myc, leading to its subsequent degradation by the proteasomes.⁷⁴ HC11 cells stably expressing c-Myc exhibits the capacity for anchorage-independent growth in soft agar and like HC11-Int3 cells, this capacity is inhibited by Gleevec. Similarly, Gleevec treatment of HC11-c-Myc tumor-bearing nude mice leads to tumor regression. The mechanism by which Gleevec inhibits c-Myc in HC11-c-Myc cells is similar to Int3 in that Gleevec has no effect on c-Myc RNA expression but is associated with absence of c-Myc protein in these cells. GSK3 β phosphorylates threonine 58 within the c-Myc Box I conserved domain, leading to c-Myc ubiquitination and proteasome-mediated degradation.⁷⁸ The data suggest that Gleevec treatment induces the ubiquitination of Int3 and c-Myc and targets them for proteasomal degradation. The effect of Gleevec on Int3 and c-Myc driven tumors taken together suggests that Gleevec treatment may be relevant for the 30% of breast cancer patients bearing c-Myc positive tumors as well as patients bearing triple negative breast tumors which frequently over express *Notch4*.^{79,80}

Authors' contributions: RC designed the study, BAC and AR generated and evaluated the data, AR contributed to overall study design and manuscript preparation.

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DECLARATION OF CONFLICTING INTERESTS

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