

# Essential functions of p21-activated kinase 1 in morphogenesis and differentiation of mammary glands

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Although growth factors have been shown to influence mammary gland development, the nature of downstream effectors remains elusive. In this study, we show that the expression of p21-activated kinase (Pak)1, a serine/threonine protein kinase, is activated in mammary glands during pregnancy and lactation. By targeting an ectopic expression of a kinase-dead Pak1 mutant under the control of ovine  $\beta$ -lactoglobulin promoter, we found that the mammary glands of female mice expressing kinase-dead Pak1 transgene revealed incomplete lobuloalveolar development and impaired functional differentiation. The expression of whey acidic protein and  $\beta$ -casein and the amount of activated Stat5 in the nuclei of epithelial cells in

transgenic mice were drastically reduced. Further analysis of the underlying mechanisms revealed that Pak1 stimulated  $\beta$ -casein promoter activity in normal mouse mammary epithelial cells and also cooperated with Stat5a. Pak1 directly interacted with and phosphorylated Stat5a at Ser 779, and both COOH-terminal deletion containing Ser 779 of Stat5a and the Ser 779 to Ala mutation completely prevented the ability of Pak1 to stimulate  $\beta$ -casein promoter. Mammary glands expressing inactive Pak1 exhibited a reduction of Stat5a Ser 779 phosphorylation. These findings suggest that Pak1 is required for alveolar morphogenesis and lactation function, and thus, identify novel functions of Pak1 in the mammary gland development.

## Introduction

Mammary gland development consists of a series of highly orchestrated events involving interactions among several distinct cell types that are regulated by complex interactions among many steroid hormones and growth factors and their receptors (Medina, 1996; Hennighausen et al., 1997; Kumar and Wang, 2002). Beside steroid hormones and their receptors, peptide growth factors and their receptors are shown to play important roles in both the morphogenesis and functional differentiation of mammary gland. For example, among the EGF receptor family receptors, EGF receptor (also known as HER1) and its ligand amphiregulin have been shown to be critical for ductal growth (Fowler et al., 1995; Xie et al., 1997; Luetke et al., 1999). In contrast, HER2, HER3, and HER4 play essential roles in the lobuloalveolar

development and functional differentiation (Niemann et al., 1998; Jones et al., 1999; Jones and Stern, 1999). Heregulin, a ligand for HER3 and HER4 receptors, is expressed during pregnancy and early lactation and has been implicated in the morphogenesis and differentiation of mammary epithelial cells (Yang et al., 1995; Jones et al., 1996). Though HER3 is a kinase-dead receptor, its heterodimerization with HER2 is important for lobuloalveolar morphogenesis (Baekstrom et al., 2000). HER4 has been shown to activate Stat5 and promote alveolar differentiation (Jones et al., 1999).

Stat5 was originally isolated from the nuclear extracts of lactating mammary gland and designated as mammary growth factor (Wakao et al., 1992). There are two separately coded isoforms of Stat5, Stat5a and Stat5b, which are highly homologous and provide functional redundancy to each other (Hennighausen and Robinson, 2001). Upon phosphorylation, Stat5 translocates to the nucleus and activates the expression of milk proteins such as  $\beta$ -casein (Hennighausen and Robinson, 2001). Stat5 could be activated by prolactin and its receptor, and by peptide growth factors and their receptors, e.g., HER4 has also been shown to activate Stat5 (Ruff-Jamison et al., 1995; David et al., 1996; Olayioye et al., 1999). However, the signaling pathway connecting growth factors to Stat5a in mammary epithelium cells remains poorly understood.

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\*Abbreviations used in this paper: BLG,  $\beta$ -lactoglobulin; DIP, dexamethasone, insulin, and prolactin; DN, dominant negative; H-E, hematoxylin and eosin; Pak1, p21-activated kinase; PCNA, proliferative cell nuclear antigen; WAP, whey acidic protein.

Key words: mammary gland; morphogenesis; Pak1; Stat5; milk proteins

One of the major protein kinases downstream of heregulin and the EGF family of receptors is the p21-activated kinase (Pak)1,\* a serine/threonine kinase (Adam et al., 1998). The Pak1 is an effector of the small GTPases Cdc42 and Rac1 (Manser et al., 1994; Nicolas and Hall, 1997) and phosphatidylinositol-3 kinase and mediates the cellular effects of polypeptide growth factors in breast cancer cells. Recent studies have shown that Pak1 regulates motility, invasiveness, anchorage-independent growth, cell survival, and angiogenesis in human breast cancer cells (Bagrodia and Cerione, 1999; Kumar and Vadlamudi, 2002). Pak1 also modulates the activation status of MAPK and p38MAPK, and thus, influences nuclear signaling (Adam et al., 2000; Vadlamudi et al., 2000a). In spite of a large number of studies in tissue culture model systems, the role of Pak1 signaling in the normal mammary development in a physiological relevant whole animal setting remains unknown and is investigated in the present study.

## Results

### Generation of DN-Pak1 transgenic mice

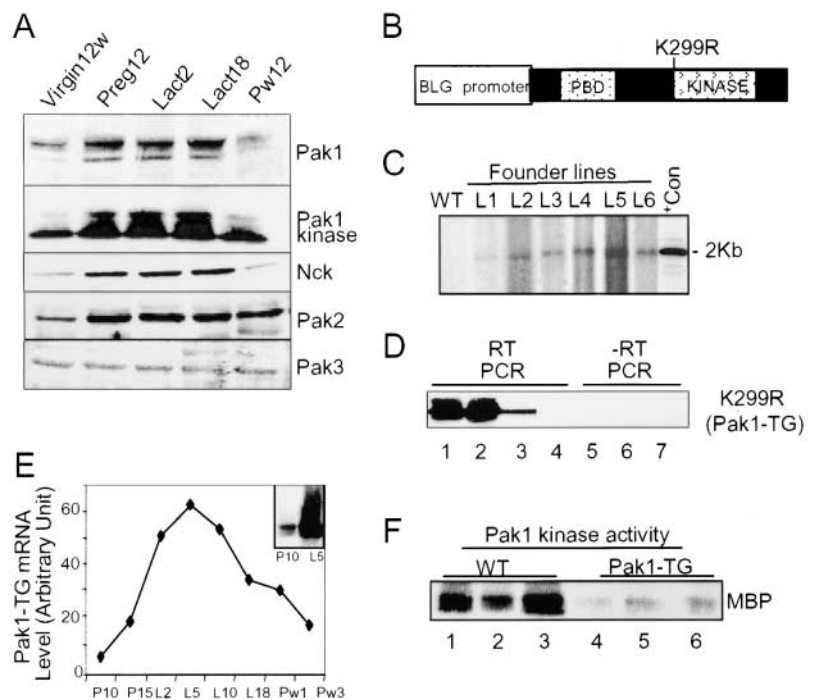
To elucidate the functional significance of the Pak1 signaling pathway in the murine mammary gland development, we first examined the Pak1 expression and kinase activity pattern. We found that Pak1 expression is easily detectable in the mammary glands, with an up-regulation of both protein level and kinase activity during pregnancy and lactation (Fig. 1 A). Accordingly, Nck, a Pak1 adaptor protein, follows the similar pattern of expression. In contrast, Pak2 and Pak3 expression is not periodically regulated (Fig. 1 A). Next, we targeted expression of a kinase-defective K299R-Pak1 mutant protein (Adam et al., 2000) (designated as dominant-negative (DN)-Pak1) during pregnancy and lactation by using the ovine  $\beta$ -lactoglobulin (BLG) promoter

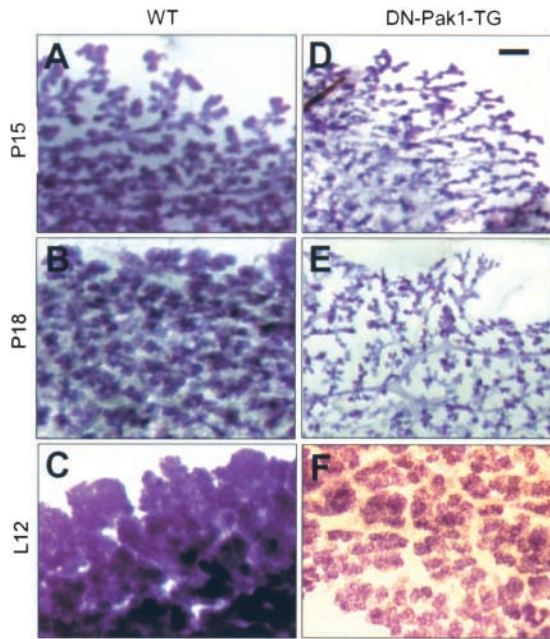
(Lundgren et al., 1997). In the targeted K299R-Pak1 sequence, the lysine 299 ATP-binding site was replaced by arginine, rendering Pak1 catalytically defective (Sells et al., 1997). Earlier studies in mammary cancer cell lines have established that the biological effects of the K299R-Pak1 in human mammary epithelial cancer cells can be mimicked by Pak1 mutant H83, 86L (which has mutations in CRIB site), and by the Pak1 autoinhibitory domain aa 83–149, and hence, were not due to ineffective transduction of Rac/cdc42 signal (Adam et al., 1998, 2000; Bagheri-Yarmand et al., 2001).

An myc-tagged kinase-dead K299R-Pak1 cDNA was cloned into the pBJ41 vector under the control of an ovine BLG promoter (Fig. 1 B). Transgenic mice having a B6DF1/J genetic background were produced by injection of purified DNA fragments into the pronucleus of fertilized mouse oocytes. The F2 and F3 generation mice were used for phenotype analysis. Among the 39 mice analyzed, we identified six DN-Pak1-TG founder mice (Fig. 1 C). Expression of the DN-Pak1 transgene was determined using RT-PCR by amplifying the region spanning the myc tag and NH<sub>2</sub> terminus of Pak1. As expected, a 345-bp transgene fragment was found in the transgenic mice but not in the wild-type mice, and the integrity of PCR band was verified using Southern blotting (Fig. 1 D, lanes 1 and 2, line 1; lane 3, line 3; lane 4, wild type). Of the six lines of DN-Pak1 transgenic mice, lines 1 and 3 had the highest level of transgene expression and were used in the subsequent analysis.

The kinetics of DN-Pak1 expression in the various stages of mammary gland development was determined using a semiquantitative RT-PCR followed by Southern blot hybridization. DN-Pak1 expression began on the tenth day of pregnancy, peaked around the second day of lactation, and returned to near the basal level, albeit detectable, at the seventh day after weaning (Fig. 1 E). This profile is in ac-

**Figure 1. Pak1 expression and kinase activity in murine mammary gland development; generation of DN-Pak1 transgenic mice.** (A) Expression and kinase activity pattern of Pak1 and its related molecules during mammary gland development. (B) Schematic diagram showing the structure of the transgene. BLG, ovine  $\beta$ -lactoglobulin promoter; PBD, p21 GTPase-binding domain. (C) Genotyping of the founder mice using Southern blotting. The tail DNA was digested with BamHI, and a transgene fragment was detected via Southern blotting. L1–L6, lines 1–6; WT, wild type; +con, positive vector control. (D) Transgene expression analysis using RT-PCR followed by Southern blotting. Lanes 1–3, DN-Pak1-TG mice on lactation day 12; lane 4, wild-type mice on lactation day 12; lanes 5–7 are from the same mice as lanes 1–3. (E) Quantitation of the transgene expression profile during mammary gland development. P, pregnancy; L, lactation; Pw, postweaning; numbers, days in a particular stage. (Insert) Transgene RT-PCR/Southern blotting at the representative stages P10 (pregnancy day 10) and L5 (lactation day 5). (F) Total lysates of mammary glands obtained from lactation day 5 mammary glands from the wild-type and Pak1-TG mice were assayed for Pak1 kinase activity using MBP as a substrate.





**Figure 2. Effect of DN-Pak1 expression on mammary gland morphogenesis.** Whole-mount staining of mammary glands obtained from wild-type and DN-Pak1-TG mice during the pregnancy and lactation stages. (A and D) In the 15th day of pregnancy, the alveoli of DN-Pak1-TG mice are less and smaller than those of wild-type mice. (B and E) In the 18th day of pregnancy, the DN-Pak1-TG mammary glands show much poorer development than the wild-type ones, and the alveoli are few and confined to the periphery of ducts, which can be viewed more clearly in Fig. 3 E. (C and F) Lactation day 12 mammary glands. Although the wild-type mammary is fully developed, DN-Pak1-TG mammary glands still have wide spaces of fat pad not filled up. Bar, 40  $\mu$ M.

cordance with the previously reported BLG-MDM2 mice (Lundgren et al., 1997). As expected, DN-Pak1 expression inhibited the endogenous Pak1 activity in lactating mammary glands (Fig. 1 F).

### DN-Pak1 expression impairs morphogenesis during pregnancy

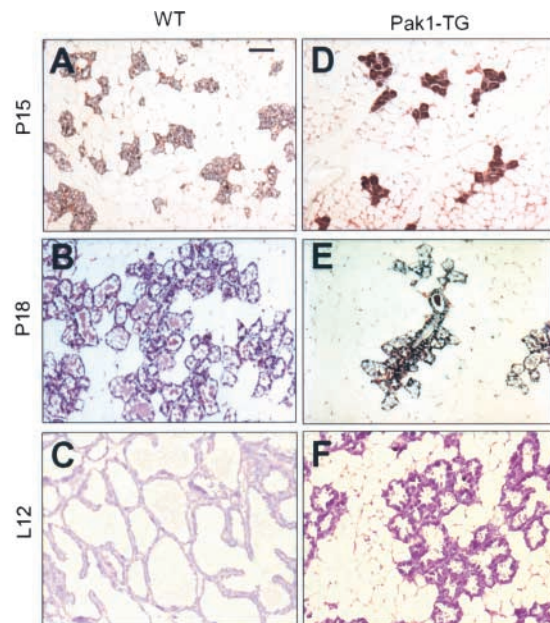
To determine the effect of Pak1 inactivation on the morphogenesis of the mammary gland, whole-mount and section hematoxylin and eosin (H-E) staining were performed. Normally, during pregnancy the mammary glands grow quickly and the ducts branch out and form lobules and alveoli. On the second day of lactation, the glandular tissues filled up the fat pad and the epithelial cells fully differentiate. During the virgin and early pregnant stages, the mammary glands of DN-Pak1-TG mice were not obviously different from those of the wild-type mice (unpublished data). However, at the late stages of pregnancy, the transgenic mammary glands showed marked dystrophy with fewer visible branches and poorly developed alveoli compared with the widespread branches and flourishing alveoli from the age-matched wild-type mammary glands (Fig. 2). Furthermore, on the 12th day of lactation, although the glandular tissues filled up the fat pad in the wild-type mammary glands, the transgenic mammary glands had distinct spaces in the absence of glandular tissues (Fig. 2, C and F). The

H-E-stained sections also demonstrated poor lobular alveolar development on the 18th day of pregnancy and moderate dystrophy during lactation (Fig. 3, B, C, E, and F).

To understand the cytological basis of the observed poor alveolar development in the mammary glands having DN-Pak1-TG expression, we analyzed the status of cell proliferation and death during the pregnancy and lactation using proliferative cell nuclear antigen (PCNA) and TUNEL staining, respectively. Mammary glands obtained from pregnant DN-Pak1-TG mice exhibited a reduced proliferation rate ( $P < 0.05$ ,  $n = 6$ ) and an elevated cell death rate ( $P < 0.01$ ,  $n = 6$ ) compared with those obtained from age-matched wild-type mice (Fig. 4. A–C).

### DN-Pak1-TG mammary glands are less functionally differentiated

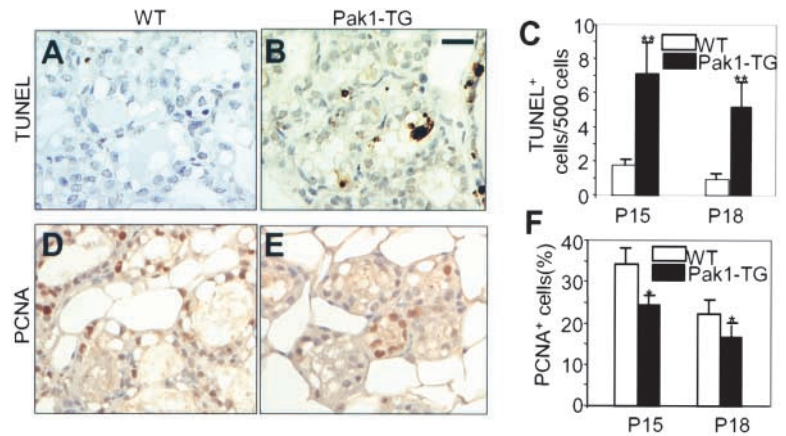
Since the DN-Pak1-TG mice exhibited impaired lobuloalveolar development, we explored whether this is accompanied by defective milk production. Indeed, the growth of the pups from and nursed by DN-Pak1-TG mothers was significantly retarded compared with those from the wild-type mothers (Fig. 5, A and B,  $P < 0.05$ ). The high level of Pak1 expression and activity during lactation and incomplete structural differentiation of lactating DN-Pak1-TG mammary glands suggested the possibility of a functional role of Pak1 in lactation. Therefore, we examined the expression of two major milk proteins,  $\beta$ -casein and whey acidic protein (WAP), by Northern blotting. The levels of both  $\beta$ -casein and WAP mRNA were significantly reduced in the mammary glands of DN-Pak1-TG mice compared with those in the age- and stage-matched wild-type mice (Fig. 5 C). To further observe that the reduced milk WAP expression in the Northern analysis was due to total reduction of alveoli epithelium or reduced WAP expression in the functional epi-



**Figure 3. H-E staining showing the fine histology of the same stages depicted in Fig. 2, the wild-type and DN-Pak1-TG glands.** Bar, 10  $\mu$ M. WT, wild type; numbers, days in a particular stage.



**Figure 4. Pak1 inactivation promotes apoptosis and slows down proliferation in pregnant mammary glands.** (A and B) TUNEL staining of mammary glands obtained from wild-type and transgenic mice at the 15th day. (C) Quantitation of the TUNEL-positive apoptotic cells in mammary glands of 15th and 18th day of pregnancy. \*\* $P < 0.01$ . (D and E) Immunohistochemical staining of PCNA in 15th day pregnant mammary glands to evaluate cell proliferation rate. (F) Quantitation of PCNA-positive cells. \* $P < 0.05$ .



thelium, we performed an in situ hybridization assay of WAP in the lactating mammary glands. Interestingly, we found a significant suppression of WAP mRNA expression in lactating mammary glands from DN-Pak1 transgenic mice compared with mammary glands from the age-matched wild-type mice (Fig. 5 D).

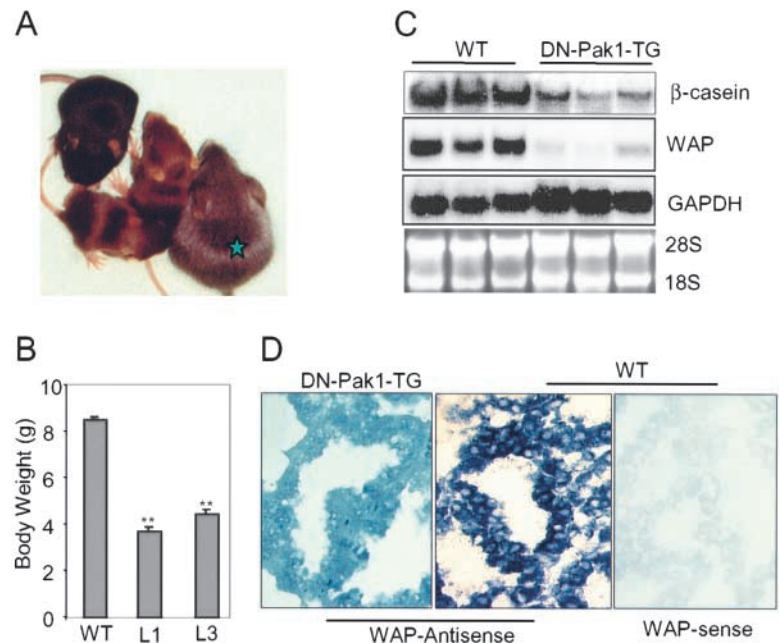
#### Pak1 stimulates $\beta$ -casein promoter in association with Stat5a

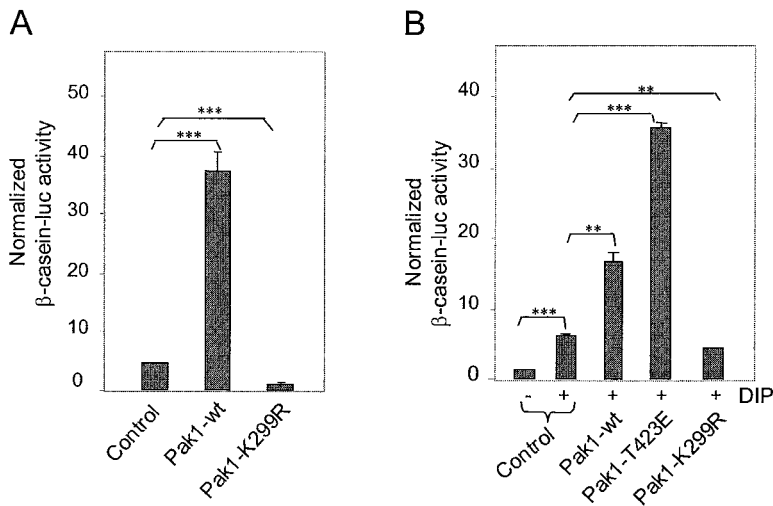
To understand the mechanism of Pak1 regulation of  $\beta$ -casein expression, we next evaluated the effects of various wild-type or mutant Pak1 on  $\beta$ -casein promoter activity using cultured HC11 normal mouse epithelial cells (Marte et al., 1995; Vadlamudi et al., 2000b). Interestingly, cotransfection with wild-type Pak1 but not DN-K299R-Pak1 significantly enhanced the  $\beta$ -casein promoter activity in the absence of exogenous hormones (Fig. 6 A). Furthermore, cotransfection of HC11 cells with wild-type or catalytically active T423E-Pak1 (Sells et al., 1997; Vadlamudi et al., 2000a) resulted in a significant potentiation of stimulation

of the  $\beta$ -casein promoter activity by lactogenic hormones, i.e., dexamethasone, insulin, and prolactin (Fig. 6 B;  $P < 0.001$ ,  $n = 6$ ). In contrast, coexpression of K299R-Pak1 mutant effectively blocked the ability of lactogenic hormones to stimulate the  $\beta$ -casein promoter activity (Fig. 6 B;  $P < 0.01$ ,  $n = 6$ ).

Earlier studies established a role for nuclear translocation of the activated transcription factor Stat5<sup>Y694-P</sup> in milk protein gene transcription and differentiation of mammary gland epithelial cells (Liu et al., 1997; Kazansky et al., 1999). To see if the DN-Pak1 overexpression induced phenotype is related to the functional status of Stat5, we next determined the status of phosphorylation and expression of Stat5. Using an anti-Stat5<sup>Y694-P</sup> antibody, we detected specific nuclear Stat5<sup>Y694-P</sup> staining in the wild-type mammary glands (Fig. 7 A), but the staining in DN-Pak1-TG mice was greatly reduced (Fig. 7 B). Consistent with these findings, mammary gland tissue lysates from DN-Pak1-TG mice contained a reduced amount of Stat5 and a further reduced amount of phosphorylated Stat5 at Y694 (Fig. 7 C).

**Figure 5. Expression of DN-Pak1 impairs milking function.** (A) Pups at the age of 15 d. The pups nursed by a transgenic mother were much smaller than that nursed by a wild-type mother (labeled with an asterisk). (B) Average body weights (in grams) of pups from lines 1 and 3 and of wild-type mothers at the age of 15 d. Bar, mean  $\pm$  SD. ( $P < 0.01$ ,  $n = 24$  each group from three litters. Each litter was standardized to 8 pups. (C) Northern blots analysis of the  $\beta$ -casein and WAP expression in three of 3-mo-old, lactation day 12 mammary glands from DN-Pak1 transgenic and wild-type mice. (D) In situ hybridization using the 12th day lactation mammary glands for WAP; sense probe was used as specificity control.





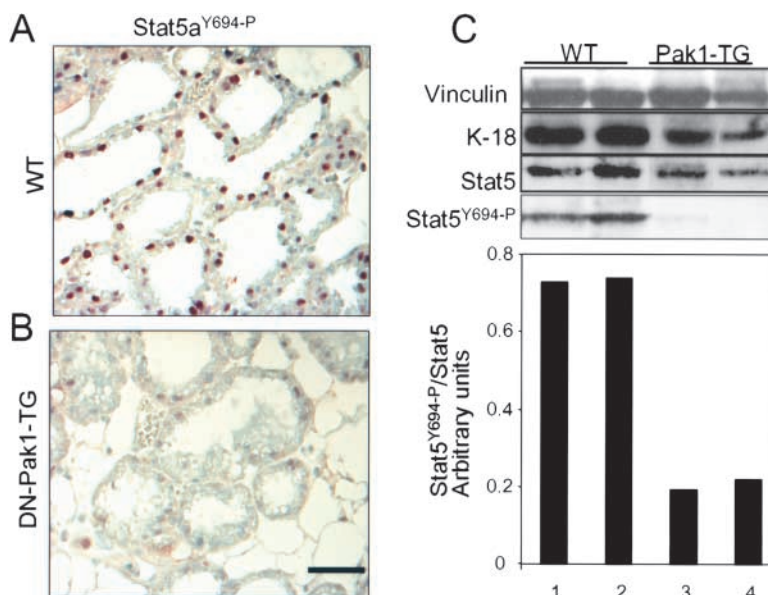
**Figure 6. Pak1 up-regulates  $\beta$ -casein promoter activity.** (A) Wild-type Pak1 but not K299R Pak1 stimulates  $\beta$ -casein promoter. HC11 cells were cotransfected with  $\beta$ -casein promoter luciferase,  $\beta$ -galactosidase, and indicated Pak1 constructs. The results are presented as normalized luciferase activity ( $***P < 0.001$ ). The experiments are repeated three times. (B) Pak1 modulates the up-regulation of  $\beta$ -casein promoter activity by hormones. HC11 cells were cotransfected with  $\beta$ -casein promoter luciferase,  $\beta$ -galactosidase, and the indicated Pak1 constructs. Cells were then treated with a mixture of dexamethasone, insulin, and prolactin (DIP) for 16 h. The results are presented as normalized luciferase activity ( $**P < 0.01$ ;  $***P < 0.001$ ). Each experiment was repeated three times.

We next wanted to delineate the signaling mechanism linking Pak1 to Stat5. We observed that a dominant-negative mutant of Stat5a with a deletion of 99 aa at the COOH-terminal (Mui et al., 1996) drastically decreased the ability of Pak1 stimulation of  $\beta$ -casein promoter-driven transcription (Fig. 8 A,  $P < 0.01$ ,  $n = 6$ ), suggesting that Stat5 is important for Pak1-mediated  $\beta$ -casein transcription. Although Tyr-694 phosphorylation of Stat5 is markedly reduced in our DN-Pak1-TG mice, it is not a Pak1 phosphorylation site and could not be the direct target of Pak1. Since Pak1 activates both the p38-MAPK and p42/44 MAPK (Vadlamudi et al., 2000a), we determined the effects of inhibiting these signaling kinases by DN p38MAPK and DN MEK, which blocks MAPK activity (Liu et al., 2001), on Pak1 induced  $\beta$ -casein transactivation. The results showed there was not any inhibitory effect (Fig. 8 B), suggesting a lack of involvement of these two signaling kinases in the noticed Pak1 regulation of  $\beta$ -casein promoter activity. Interestingly, catalytically active T423E-Pak1 mutant drastically stimulated  $\beta$ -casein promoter when cotransfected with wild-

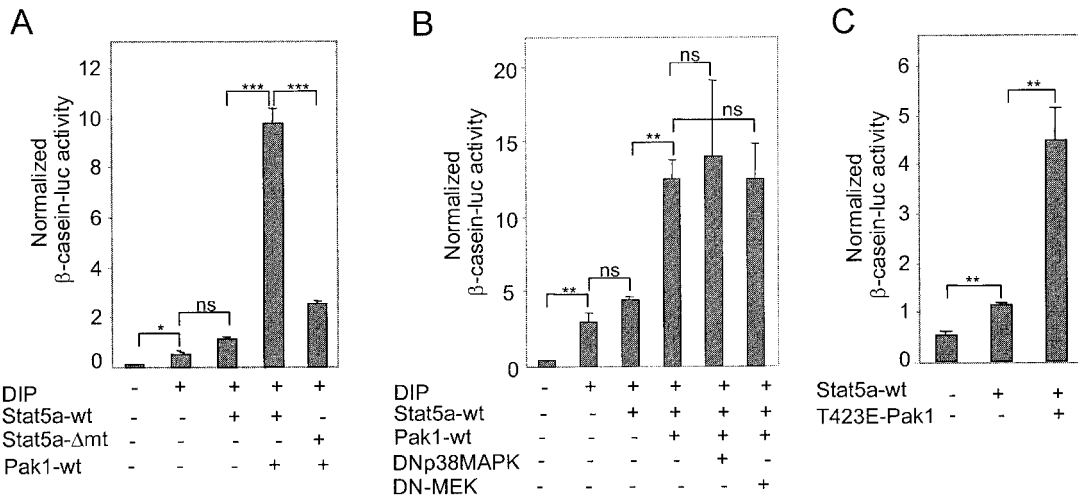
type Stat5a in the absence of lactogenic hormones (Fig. 8 C). These findings raised the possibility that Pak1 may directly phosphorylate Stat5a.

**Pak1 interacts with and phosphorylates Stat5a**

To implicate a role of Pak1 signaling in Stat5a activation, we first examined if Pak1 interacts with Stat5a in GST pull-down assay using  $^{35}$ S-labeled Pak1 and purified GST-Stat5a fusion protein. Results show that GST-Stat5a but not GST alone interacted with Pak1 (Fig. 9 A). Since Stat5a is constitutively phosphorylated at the Ser 779 ( $S^{779}$ ) site, and the levels of Stat5a-Ser 779 phosphorylation are specifically high in late pregnancy and early lactation, and because the kinase, which phosphorylates this site, is unknown (Beuvink et al., 2000), we hypothesized that Pak1 may represent a potential kinase for Ser 779 phosphorylation of Stat5a. A careful examination of Stat5a structure suggested that, indeed, Stat5a Ser 779 could serve a Pak1 consensus site. The phosphorylation site of Stat5a Ser 779 has sequence of ARLS containing one basic charge in -3 similar to some of the



**Figure 7. DN-Pak1-TG mammary glands show decreased Stat5 tyrosine phosphorylation and expression.** (A and B) Immunohistochemical staining of phospho-Stat5a in mammary glands obtained from wild-type and Pak1-TG mice on lactation day 5. (C) Effect of Pak1 inactivation on the levels of Stat5a and phosphorylated Stat5a by immunoblot analysis. Bar: (A and B) 20  $\mu$ m.



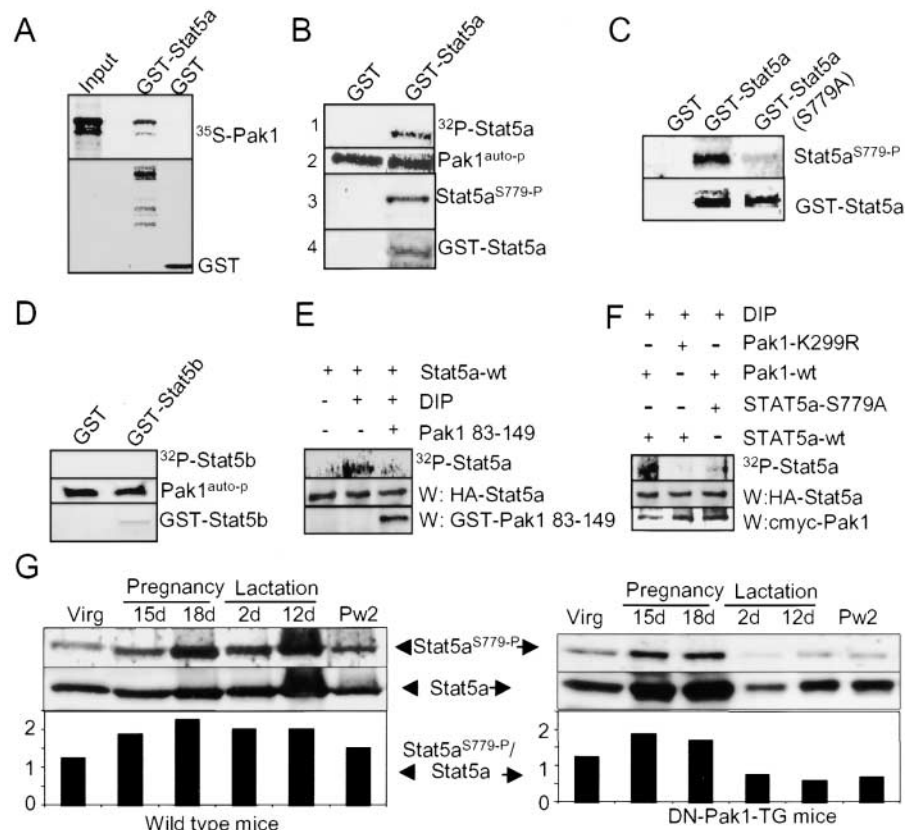
**Figure 8. Pak1 stimulates the β-casein promoter activity by interacting with Stat5a.** HC11 cells were cotransfected with β-casein promoter luciferase and the indicated constructs, treated with or without lactogenic hormones. (A) Pak1 stimulates β-casein promoter activity when cotransfected with wild-type Stat5a, but this stimulation is almost gone when cotransfected with mutated Stat5a, which has a COOH-terminal deletion including the site of Ser 779. (B) Pak1 stimulation of β-casein promoter cannot be blocked by the dominant-negative forms of p38MAPK or MEK. (C) Constitutively active T423E-Pak1 strongly stimulates β-casein promoter when cotransfected with wild-type Stat5a without DIP treatment. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001; ns, P > 0.05, not significantly different.

other Pak phosphorylation sites found in MEK1 and Vimentin. Thus, we next tested whether Stat5a is a substrate of Pak1. Results show that Pak1 enzyme directly phosphorylates Stat5a (Fig. 9 B). Interestingly, Pak1-mediated phosphorylation of Stat5a was also readily detected with an anti-

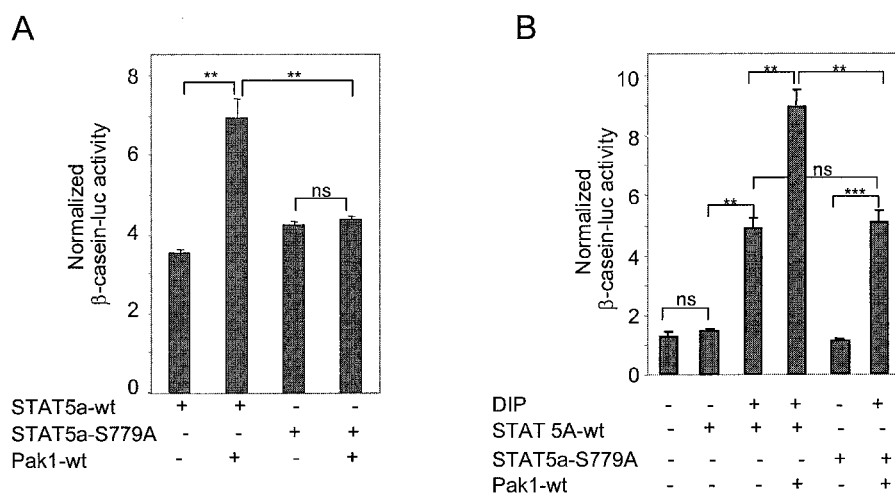
body (Beuvink et al., 2000) that specifically recognizes phosphorylated Stat5a on Ser 779 (Fig. 9 C). To validate that Ser 779 on Stat5a was a bona fide Pak1 phosphorylation site, we next used the Stat5a mutant with Ser 779 to Ala (S779A) substitution. As we expected, Pak1 failed to

**Figure 9. Pak1 directly interacts and phosphorylates Stat5a in vitro.**

(A) <sup>35</sup>S-labeled Pak1 TNT products were pulled down by GST-Stat5a but not GST alone. (B) GST-Stat5a protein was incubated with Pak1 at the presence of [γ-<sup>32</sup>P]ATP and resolved on an SDS-PAGE gel. A clear band at the size of GST-Stat5a showed up by autoradiography (panel 1), and the same band is also recognized by blotting with Ser 779-phospho-specific Stat5a antibody (panel 3). (C) The experiment was done the same way as in B, but the phospho-Stat5a band cannot be detected with the S779A mutant GST-Stat5a. (D and E) HC11 cells were transfected with indicated expression constructs, and treated with or without DIP, serum starved, and labeled with <sup>32</sup>P-orthophosphate overnight. Cells were lysed, immunoprecipitated with HA antibodies, and resolved on SDS-PAGE developed by autoradiography. The same cell lysates were also checked for the expression of transfected plasmids by direct Western blotting. (F) Phosphorylation status of Stat5a<sup>S779</sup> in different developmental stages of wild-type versus DN-Pak1-TG mammary glands. Western blotting was done with anti-Stat5a<sup>S779</sup> in the presence of nonphospho absorption peptide (Beuvink et al., 2000) and antibodies against total Stat5a. The ratio of Stat5a<sup>S779</sup> was normalized with the virgin stage as 1.







**Figure 10. Pak1 stimulates the  $\beta$ -casein promoter activity by phosphorylating Stat5a at the Ser 779 site.** HC11 cells were cotransfected with  $\beta$ -casein promoter luciferase and the indicated constructs, treated with or without lactogenic hormones. (A) Pak1-stimulated  $\beta$ -casein promoter was blocked by the S779A mutation of Stat5a. (B) The S779A mutant responded to lactogenic hormone treatment equally as wild-type Stat5a in stimulating  $\beta$ -casein promoter activity, but Pak1 cannot further increase it as to wild-type Stat5a. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; ns,  $P > 0.05$ , not significantly different.

phosphorylate the S779A-Stat5a mutant (Fig. 9 C). Since Stat5b lacks the Ser 779 site, as an additional control, the Pak1 enzyme failed to phosphorylate Stat 5b (Fig. 9 D), suggesting that Pak1 specifically phosphorylates the Ser 779 site in Stat5a.

To examine the effect of Pak1 phosphorylation on Stat5a in vivo, HC11 cells were cotransfected with Pak1 and/or Pak1 inhibitory fragment aa 83–149 (Bagheri-Yarmand et al., 2001), wild-type or mutant HA-tagged Stat5a, treated with or without dexamethasone, insulin, and prolactin (DIP), and cells were metabolically labeled with  $^{32}\text{P}$ -ortho-phosphoric acid. Cell lysates were immunoprecipitated with an anti-HA mAb and analyzed by autoradiography. Results indicated that transfected Stat5a was phosphorylated in response to DIP treatment, and this phosphorylation could be blocked by the expression of Pak1 aa 83–149 inhibitory fragment (Fig. 9 E). Further experiments showed that Stat5a phosphorylation was also blocked by both K299R Pak1 and S779A Stat5a mutant (Fig. 9 F). These results further validate the physiological significance of Pak1 phosphorylation of Stat5a at Ser 779.

Pak1 stimulates  $\beta$ -casein promoter activity through phosphorylation of Stat5a at Ser 779 with the establishment of Ser 779 of Stat5a as the major phosphorylation site by Pak1. We next evaluated if mutation of this site will also block Pak1-mediated stimulation of  $\beta$ -casein promoter activity similar to the inhibition obtained by a truncated Stat5a mutant in Fig. 8 A. Not surprisingly, S779A mutation of Stat5a also totally blocked the Pak1 stimulation of  $\beta$ -casein promoter activity with or without DIP treatment (Fig. 10, A and B).

## Discussion

By using a DN-Pak1 mutant under the control of ovine BLG promoter, which is expressed during pregnancy and lactation, we have shown that targeted inactivation of Pak1 in mammary epithelial cells leads to severe impairment of normal development of mammary glands. In particular, Pak1 signaling is required for lobuloalveolar development and lactation functions during mammary gland morphogenesis. At least one of the underlying mechanisms includes Pak1-mediated direct phosphorylation of the Ser 779 site

of Stat5a transcription factor, leading to stimulation of  $\beta$ -casein promoter activity.

Mammary gland development can be divided into phases of ductal development and lobuloalveolar development. Ductal development is achieved after puberty without pregnancy. Usually, in 12-wk-old virgin mice ductal branches fill the whole mammary pad. And the lobuloalveolar development is dependent on pregnancy and requires pregnancy-linked signals. Among a bunch of signals, prolactin-Jak2-Stat 5 pathways are believed to play key roles in alveolar development and differentiation. Null mutation of any one of these three molecules has been found to result in almost complete loss of alveolar development (Miyoshi et al., 2001; Shillingford et al., 2002). However, several other molecules, including growth factors and protein kinases, are also up-regulated during pregnancy and lactation and have been shown to be involved in alveolar development or milking (Chodosh et al., 2000). For example, Stat5 cannot only be activated through the prolactin receptor but also by growth hormones and EGF receptors in mammary epithelia (Gallego et al., 2001). Moreover, the prolactin receptor also stimulates phosphatidylinositol 3-kinase pathway, a known upstream activator of Pak1 signaling (Adam et al., 1998). Pak1-mediated signal in alveolar development may not be related to the phosphorylation of Stat5a, since the null mutation of Stat5a did not display a defect in alveolar development but rather in the functional differentiation (Liu et al., 1997). The mechanism of Pak1 regulation of the alveolar morphogenesis might involve its role in supporting cell survival (Schurmann et al., 2000) and promoting cell proliferation (Howe and Juliano, 2000; Vadlamudi et al., 2000a), since we noticed an increased apoptosis and a decreased proliferation rate of epithelial cells in the pregnant DN-Pak1-TG mammary glands.

We also considered the possibility of whether Pak1 inhibition-associated disruption of the cytoskeleton might contribute to the noticed phenotypes. However, our data does not support this possibility, since there was no significant difference in the status of actin staining in mammary glands from transgenic and wild-type mice (Fig. S1, available at <http://www.jcb.org/content/full/jcb.200212066/DC1>). It is possible that the lack of effect of Pak1 on cytoskeletal reor-

ganization in the transgenic mice could be due to the fact that Pak1 affects cytoskeleton via kinase-dependent and -independent functions (Sells et al., 1997; Adam et al., 2000; Vadlamudi et al., 2000a). Kinase-dependent functions are implicated in dissolution of stress fibers, whereas kinase-independent functions are implicated in generation of lamellipodia and ruffling via protein-protein interactions. In addition, Pak2 and Pak3 whose kinase domain is highly conserved to Pak1 is highly expressed in mammary gland and may compensate the Pak1 kinase activity, which is required for reorganization of cytoskeleton.

Stat5a was originally isolated from pregnant mammary gland (Wakao et al., 1992), named mammary gland growth factor, and is shown to play a role in the normal morphogenesis of mammary gland by supporting cell survival and stimulating epithelial cell proliferation. Indeed, the Stat5a-null mutation in mice on a large scale blocked the alveolar development of mammary gland during pregnancy, with a marked reduction in cell proliferation index of alveolar cells. Therefore, a full functional activation of Stat5a might be required for the normal mammary gland development. In brief, our findings of a close phenotypic resemblance between DN-Pak1-TG mutant Stat5a mice and that of Pak1 phosphorylated Stat 5a, reduction in the Stat5a phosphorylation in Pak1 mutant mice, and Pak1 regulation of transcriptional functions of Stat 5a strongly suggest that Pak1 regulates Stat5a pathway, and in principle, Stat 5a might act as a downstream target of upstream activators of Pak1. Therefore, Pak1 regulation of Stat5a might play a role in morphogenesis and differentiation of the mouse mammary gland.

The process of mammary gland differentiation is profoundly influenced by Stat5 transactivation functions (Hennighausen et al., 1997). Tyrosine phosphorylation of Stat5, both a/b isoforms at site Y694/699, is critical for the milking function of mammary epithelia. In the present study, the inability of transgenic lactating DN-Pak1-TG mice to nurse pups is partly due to the impaired alveolar development. However, drastic reduction in  $\beta$ -casein and WAP expression indicated an impairment of the functional differentiation of existing alveolar epithelia. In fact, the morphology of DN-Pak1-TG mammary alveoli was distinctly less differentiated with a reduced level of Stat5 expression and its phosphorylation at Y694/699. Our data from normal mammary epithelial HC11 cells also suggested that Pak1 stimulates  $\beta$ -casein promoter activity in a lactating hormone-independent manner. Moreover, K299R Pak1 blocks the basal  $\beta$ -casein promoter activity, highlighting the significance of Pak1 activity in the functions of mammary glands. Immunohistochemical staining and Western blotting showed that DN-Pak1-TG mammary glands exhibited both reduced expression and decreased tyrosine phosphorylation of Stat5. Although these data help us understand the impairment of mammary gland functions, the mechanism of this regulation is still yet to be determined.

Aside from tyrosine phosphorylation, Stat5 is also phosphorylated on two other serine sites, Ser 725 (Stat5a)/Ser 730 (Stat5b) and Ser 779, which only exist in Stat5a (Yamashita et al., 1998, 2001; Beuvink et al., 2000); however,

the kinase which phosphorylates these two sites are not known to date. Here we provided evidence that Pak1 directly interacts and phosphorylates Stat5a at Ser 779. We also demonstrated that mutation of Ser 779 to Ala blocks the ability of Pak1 to phosphorylate Stat5a, and consequently, stimulation of  $\beta$ -casein promoter activity in physiological relevant murine normal mammary epithelial HC11 cells. These findings are significant, since earlier studies using COS or MCF-7 cells did not notice any inhibitory effect of Stat5a Ser 779 Ala on the stimulation of  $\beta$ -casein promoter activity by prolactin (Beuvink et al., 2000; Yamashita et al., 2001). It is also possible that prolactin signaling does not target Ser 779 phosphorylation, since prolactin is not an effective inducer of Pak1 activity in HC11 cells (unpublished data). In summary, results presented here have shown a role of Pak1 signaling in the Stat5a Ser 779 phosphorylation and transactivation of  $\beta$ -casein promoter and have shown that targeted inactivation of Pak1 in mammary epithelial cells leads to severe impairment of normal mammary gland development.

## Materials and methods

### Transgenic mice and genotyping

An myc-tagged kinase-dead Pak1 cDNA containing lysine to arginine replacement at position 299 (K299R) (Sells et al., 1997) was cloned into the pBJ41 vector under the control of an ovine BLG promoter (Lundgren et al., 1997) at the site of BamHI. Digestion with FspI released an 8.5-kb fragment from the vector. The fragment was purified on a 0.8% low melting agarose gel by cutting out the transgenic fragment and digesting it with Gekase. The DNA was then purified by Resource Q anion exchange FPLC, and ethanol was precipitated. The fragment was resuspended in 10 mM Tris (pH 7.4) and 0.1 mM EDTA at a final concentration of 3 ng/ $\mu$ l. The construct was injected into the pronucleus of fertilized B6DF1/J mouse oocytes and transferred back to pseudopregnant mice uterus (injection and oocytes transfer were done by Chad Smith [Transgenic Core Facility of M.D. Anderson Cancer Center]). The founder mice were genotyped via Southern blotting with 5  $\mu$ g of tail DNA digested with BamHI. The size of the transgene band is 2.0 kb. PCR genotyping was performed to amplify a 345-bp sequence between the myc tag and Pak1 cDNA using the following primers: forward, 5'-CATCTCTGAAGAGGATCTTGCTAGCG-3' and reverse, 5'-GGGTTTTCTCTGCTCCGACTTA-3'.

### Whole-mount and H-E staining

The whole left no. 4 mammary gland of each animal was removed and mounted on slides. After drying for 10–30 min, the tissues were fixed with acetic acid/ethanol (1:3) for 1 h. Then, the tissues were rinsed with water briefly and stained with 0.2% carmine/0.5% aluminum potassium sulfate overnight. Tissues were then dehydrated through graded ethanol, defatted through acetone, cleared, and preserved in methyl salicylate. The right no. 4 mammary glands were fixed with 10% formaldehyde, processed to paraffin sections, and stained with H-E.

### Kinase assay

Pak1 assay was performed as reported previously (Adam et al., 1998). 1 mg of total protein lysate was immunoprecipitated with an anti-Pak1 antibody (sc-882; Santa Cruz Biotechnology, Inc.) and subjected to an *in vitro* complex kinase assay using myelin basic protein as a substrate for 30 min at 30°C. The reaction products were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane. The membrane was exposed to a phosphoscreen overnight and scanned with Storm PhosphorImager.

### Immunohistochemistry and TUNEL staining

Immunohistochemical staining of PCNA (P8825; Sigma-Aldrich), Y<sup>694</sup>-phospho-Stat5 (1:50; 71–6900; Zymed Laboratories) was done with the indirect enzyme labeling method as described previously (Wang et al., 1998). Antigen retrieval was performed by boiling the sections for 10 min and gradually cooling them down for 30 min in 0.01 M, pH 6.0, citrate buffer before incubating with the antibody. TUNEL staining was also performed as described previously (Wang et al., 1998).



### RT-PCR, Northern blot, and immunoblotting

RT-PCR was performed using the Access RT-PCR system (Promega) per the manufacturer's instructions. Before performing RT-PCR, the total RNA was first digested with Q1 DNase for 10 min to get rid of possible genomic DNA contamination. For Northern blot analysis, 20 µg of total RNA was resolved on a 1% formaldehyde agarose gel, transferred to a nylon membrane, and probed with appropriate probes and exposed to phosphor-screen. For immunoblot analysis, 200 µg of the total protein lysate was resolved on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and probed with appropriate antibodies.

### Expression vectors

Wild-type Pak1, K299R-Pak1 and T423E-Pak1 (Adam et al., 1998), and GST-S779A-Stat5a (Beuvink et al., 2000) plasmids have been described previously. β-casein promoter-luciferase vector was obtained from Dr. J. Rosen (Baylor College of Medicine, Houston, TX), DNp38-MAPK was from Dr. S. Ludwig (University of Wurzburg, Wurzburg, Germany).

### Cell culture, transfection, and luciferase assay

HC11 mouse epithelial cells were maintained in RPMI-1640 medium supplemented with 8% FCS, 10 ng EGF/ml, and 5 µg insulin/ml as described previously (Vadlamudi et al., 2000b). Cells were plated in six-well culture plates, and transfection was performed using the Fugene (Roche) method. Luciferase assay was performed according to the manufacturer's instructions as described previously (Mazumdar et al., 2001) and results were standardized against β-gal luciferase of internal control. Each experimental group includes three triplicate plates.

### GST pull-down assays

In vitro transcription and translation of the Pak1 were performed using the TNT transcription-translation system (Promega) as described (Mazumdar et al., 2001). In brief, the appropriate cDNAs (1 µg) were translated in vitro in the presence of <sup>35</sup>S-methionine in a reaction volume of 50 µl using a T7-TNT kit (Promega). The reaction mixture was diluted to 1 ml with NP-40 lysis buffer (25 mM Tris, 50 mM NaCl, 1% Nonidet P40). A 250-liter aliquot was used for each GST pull-down assay. Translation and product size were verified by analyzing 2 µl of the reaction mixture with SDS-PAGE and autoradiography. GST pull-down assays were performed by incubating equal amounts of GST and GST immobilized on GST beads (Amersham Biosciences) with in vitro-translated <sup>35</sup>S-labeled Pak1. After incubating for 2 h at 4°C, the beads were washed five times with NP-40 lysis buffer, eluted with 2× SDS buffer, and resolved on SDS-PAGE and revealed by autoradiography.

### In vitro and in vivo phosphorylation assays

In vitro and in vivo phosphorylation assay was done as described previously (Vadlamudi et al., 2002). Briefly, for in vitro assay the GST-Stat5a fusion protein was incubated in 50 mM Hepes, 10 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, and 1 mM dithiothreitol containing 1 µg of purified bacterially expressed GST-Pak1 enzyme, 10 µCi of [γ-<sup>32</sup>P]ATP and 25 M cold ATP. The reaction was performed in a volume of 30 µl for 30 min at 30°C and then stopped by adding 10 µg of 4× SDS sample buffer, resolved on SDS-PAGE gel, and revealed by autoradiography. For in vivo phosphorylation assay, HC 11 cells were transfected with wild-type or S779A mutant Stat5a, wild-type Pak1 or Pak1 83–149 inhibitory peptide, or K299R Pak1. After 36 h, the cells were labeled with <sup>32</sup>P-orthophosphate overnight (0.2 mCi/ml) and treated with DIP for 30 min. Cells were lysed, and equal amounts of the protein were immunoprecipitated with an antibody specific for HA tag to immunoprecipitate HA-STAT or HA S779A Stat. An aliquot of total lysate was run as a separate gene gel to analyze the expression of GST-Pak inhibitory fragment aa 83–149 and myc-tagged Pak constructs.

### In situ hybridization

In situ hybridization was done as we described previously (Wang et al., 2002). Mouse mammary glands were fixed with 4% PFA, and frozen sections were cut. A 419 bp of mouse WAP cDNA was amplified by RT-PCR, subcloned into TOPO II vector (In Vitrogen), and used for riboprobe synthesis under the control of T7 promoter. Primers used are forward, 5'-CCT-GACACCGGTACCATGCGTTGC-3' and reverse, 5'-CACTGAAGGGT-TATCACTGGCACT-3'; RNA probes were labeled with digoxigenin (Roche) and hybridized for 16–20 h in buffer containing 0.1 µg/ml riboprobes, 50% formamide, 300 mM NaCl, 10 mM Tris (pH 7.4), 10 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 6.8), 5 mM EDTA (pH 8.0), 0.2% Ficoll 400, 0.2% polyvinyl pyrrolidone, 10% dextran sulfate, 200 µg/ml yeast total RNA, and 50 mM dithiothreitol. Alkaline phosphatase-labeled sheep antidigoxigenin anti-

body was applied, and signals were visualized by NBT-BCIP. Hybridization with sense probe was used as background control.

### Quantitation and statistical analysis

Counting of PCNA and TUNEL-positive cells was done manually on the computer screen for five fields of each case. Statistical analysis for pups weight, and PCNA and TUNEL staining and β-casein promoter luciferase assay were done by Student's *t* test. Semiquantitation of Western blotting was performed by using the SigmaGel software.

### Online supplemental material

Fig. S1 showing phalloidin staining of actin in mammary glands from wild-type and DN-Pak1-TG mice is available at <http://www.jcb.org/content/full/jcb.200212066/DC1>.

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