Breast cancer is the most common form of cancer in women. Despite significant therapeutic advances in recent years, breast cancer also still causes the greatest number of cancer-related deaths in women, the vast majority of which (> 90%) are caused by metastases. However, very few mouse mammary cancer models exist that faithfully recapitulate the multistep metastatic process in human patients. Here we assessed the suitability of a syngrafting protocol for a Myc-driven mammary tumor model (WAP-Myc) to study autochthonous metastasis. A moderate but robust spontaneous lung metastasis rate of around 25% was attained. In addition, increased T cell infiltration was observed in metastatic tumors compared to donor and syngrafted primary tumors. Thus, the WAP-Myc syngrafting protocol is a suitable tool to study the mechanisms of metastasis in MYC-driven breast cancer.
recaptulates the course of the metastatic disease in the clinic. However, it should be noted that the development of metastatic tumors takes a long time (2–6 months), making routine applications time-consuming and costly. Nevertheless, applying this approach in other GEMMs of BCa could yield metastatic models of breast cancers driven by diverse genetic changes, increasing our understanding of metastasis in different contexts.

C-MYC is frequently amplified and/or overexpressed in human breast cancers and is associated with poor outcome and metastasis. Through regulation of critical cellular processes, including ribosome biogenesis, translation, cell cycle and metabolism, MYC overexpression confers a growth advantage to tumor cells. As MYC-overexpressing tumor cells depend on MYC for survival and proliferation, MYC has been a compelling target for therapeutic intervention. Although targeting MYC directly has been difficult as it is a transcription factor without enzymatic activity, several attempts have been made. As an alternative, new strategies have been developed to target MYC-driven oncogenic processes. For example, using a transgenic model for MYC-driven mammary cancer (the WAP-Myc mouse model), we recently identified MYC-driven anti-apoptotic pathways as a vulnerability in MYC-high breast cancer. In the WAP-Myc mouse model, MYC is overexpressed in the mammary gland under the whey acidic protein (Wap) promoter resulting in the development of breast adenocarcinomas with a latency of 2–3 months. However, while WAP-Myc mice are an excellent model to investigate therapeutic approaches against MYC, their use has been limited to studies of primary tumors as WAP-Myc tumors rarely metastasize.

In this study, we investigated whether the WAP-Myc model can be developed into a model for spontaneous metastasis of MYC-driven breast cancer by applying a recently developed protocol for WAP-Myc syngrafting and resection of the primary tumor generated from syngrafted cells.

**Material and methods**

**Mice.** WAP-Myc mice (FVB.Cg-Tg(Wap-Myc)212Bri/J) and Luciferase-GFP reporter mice FVB-Tg(CAG-luc,-GFP)2L2G85Shco/J were obtained from the Jackson Laboratory (Bar Harbor, Maine, United States). Genotyping was conducted by PCR analysis following the protocols of the Jackson Laboratory. To generate the FVB/N-tg(Wap-Myc; CAG-luc,-GFP) transgenic mice, 8–10 weeks old WAP-Myc males and Luc;GFP females were crossed and the heterozygous offspring from different crossings were bred to obtain homozygous Wap-Myc;Luc;GFP transgenic mice. 4-week-old, wild-type FVB mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France). All experimental procedures involving animals were approved by the National Animal Ethics Committee of Finland (License number: ESAVI/3678/04.10.07/2016), and mouse colonies were maintained in accordance with the protocols of the Experimental Animal Committee of the University of Helsinki.

**Isolation of donor tumor cells.** To induce MYC expression under the Wap promoter, WAP-Myc or WAP-Myc;Luc;GFP females underwent two pregnancies, after which mice were monitored weekly for mammary tumor formation. When tumors were measured to be approximately 10 mm × 10 mm in dimensions, tumors were harvested into an ice-cold solution of PBS. In a laminar hood, PBS was removed and tumors were mechanically minced using sterile scalpels. Tumor pieces were then transferred into DMEM/F12 medium containing 5% FCS, 200 mM L-glutamine, 5 μg/ml insulin, 50 μg/ml gentamycin and 0.2% collagenase A (w/v) and incubated at 37 °C for 2 h with moderate shaking (120 rpm). Samples were washed four times with DMEM/F12 medium without collagenase A and digested further with trypsin for 10 min at 37 °C. Following a wash with complete DMEM/F12 medium, cells were counted and either stored frozen in liquid nitrogen, cultured overnight or transduced with a lentiviral vector to express luciferase. Lentiviral particles were prepared using the pLenti CMV Puro LUC (w168-1) plasmid which was a gift from Eric Campeau and Paul Kaufman (Addgene plasmid #17477; https://n2t.net/addgene:17477; [23]).

**Orthotopic transplantation of tumor cells.** 4-week-old FVB female recipient mice were injected with painkillers (carprofen and buprenorphine) and anesthetized using inhaled 2.5% isoflurane. After disinfection, a midline incision was made and the 4th mammary glands were exposed. 25,000–100,000 WAP-Myc tumor cells, resuspended in 10 μl PBS containing 1% FBS, were injected bilaterally into the 4th mammary glands and the skin was sealed with wound clips. The mice were monitored after surgery to ensure well-being.

**Surgical resection of tumors.** Primary tumor size was measured three times a week using digital calipers. Tumor volumes were calculated using the formula V = d2 × D/2, where d represents the shortest diameter and D represents the longest. When one of the bilateral tumors reached 750 mm3 in volume, both tumors were surgically resected under isoflurane anesthesia and stored in liquid nitrogen. The incision was closed using wound clips. The mice were monitored after surgery to ensure well-being. After surgery, the mice were followed for secondary tumor formation or signs of metastatic disease. Mice were sacrificed either when the diameter of the secondary tumors reached 1.5 cm or when they showed signs of metastatic disease, as indicated by difficulty breathing or weight loss.

**Tumor immunoprofiling.** 0.1 g of the resected tumors was digested in DMEM/F12 medium containing 2 mg/ml collagenase A for 1.5 h at 37 °C on a shaker. After washing, samples were further digested in TrypLE express (12604013, ThermoFisher) for 10 min at 37 °C. Samples were filtered through a 70 μm cell strainer, split into three separate tubes and stained for 15 min in room temperature with the antibody panels listed in Table 1. Samples stained with antibody panel 2 were fixed and permeabilized using Fixation/Permeabilization Solution kit (BD) as some of the target proteins are intracellular.

The samples were analyzed using FACSVerse (BD) and analyzed using Flowjo version 10.4. To analyze panels 1 and 2, cells were first gated for singlets, then CD45 + leukocytes were selected. Leukocytes were assessed by the expression of CD3 and NK1.1 to identify T cells (CD3 + NK1.1−), NK cells (NK1.1+ , CD3−) and NKT
burden. In an attempt to increase the frequency of metastasis, WAP-Myc tumor cells were orthotopically grafted into recipient mice and, to give more time for metastatic tumors to develop, primary tumors were surgically resected when one of them reached 750 mm³, which took 5–6 weeks for tumors grown from fresh cells and 8–13 weeks for tumors from frozen cells (Fig. 1a), similar to the approach taken by Doornebal et al. for the conditional K14cre;Cdh1F/F;Trp53F/F mouse model. Throughout the remainder of the paper, for syngrafting experiments the term primary tumor is used to indicate the primary tumor generated from syngrafted cells and the term donor tumor indicates the tumor the syngrafted cells were derived from.

To develop a metastatic mouse model of MYC-driven breast cancer, we used transgenic WAP-Myc mice, which develop breast adenocarcinomas following two pregnancies. As these mammary tumors grow relatively fast, metastatic tumors are rare when the mice are sacrificed due to primary tumor burden. In an attempt to increase the frequency of metastasis, WAP-Myc tumor cells were orthotopically syngrafted into recipient mice and, to give more time for metastatic tumors to develop, primary tumors were surgically resected after reaching a volume of 750 mm³ (Fig. 1a), similar to the approach taken by Doornebal et al. for the conditional K14cre;Cdh1F/F;Trp53F/F mouse model. Throughout the remainder of the paper, for syngrafting experiments the term primary tumor is used to indicate the primary tumor generated from syngrafted cells and the term donor tumor indicates the tumor the syngrafted cells were derived from.

To establish the protocol we performed a preliminary experiment where either 100,000 fresh (never frozen) or 200,000 frozen-and-thawed donor tumor cells were syngrafted into the fat pads of both 4th mammary glands in recipient mice (eight mice in total). Donor tumor cells were transduced with CMV-Puro-Luc vector prior to implantation to monitor primary and metastatic tumor growth but, while bioluminescence signals could be detected for primary tumors, we could not detect them for metastatic tumors (data not shown). Primary tumors were resected when one of them reached 750 mm³, which took 5–6 weeks for tumors grown from fresh cells and 8–13 weeks for tumors from frozen cells (Fig. 1b). One mouse had to be sacrificed before any signs of primary tumor growth and was eliminated from analysis. After tumor resection, mice were sacrificed upon growth of spontaneous lung metastases with a diameter of 15 mm or signs of discomfort due to metastasis, after which the mice were surveyed for metastasis in the lungs, liver, brain, lymph nodes and spleen. Spontaneous lung metastases were detected in two out of seven mice, both transplanted with frozen-and-thawed cells (Fig. 1b,c). None of the mice developed detectable metastases in other organs.

Despite the low number of mice, this preliminary experiment suggests that in this setup frozen-and-thawed donor tumor cells are more likely to form metastases than fresh donor tumor cells (Fig. 1b). The delay in primary tumor growth with frozen-and-thawed cells (Fig. 1b) could provide a longer window for metastatic cells to disseminate and for metastases to form.

### Results

**Development of the transplantation and tumor resection protocols for the WAP-Myc metastatic mouse model.** To develop a metastatic mouse model of MYC-driven breast cancer, we used transgenic WAP-Myc mice, which develop breast adenocarcinomas following two pregnancies. As these mammary tumors grow relatively fast, metastatic tumors are rare when the mice are sacrificed due to primary tumor burden. In an attempt to increase the frequency of metastasis, WAP-Myc tumor cells were orthotopically syngrafted into recipient mice and, to give more time for metastatic tumors to develop, primary tumors were surgically resected after reaching a volume of 750 mm³ (Fig. 1a), similar to the approach taken by Doornebal et al. for the conditional K14cre;Cdh1F/F;Trp53F/F mouse model. Throughout the remainder of the paper, for syngrafting experiments the term primary tumor is used to indicate the primary tumor generated from syngrafted cells and the term donor tumor indicates the tumor the syngrafted cells were derived from.

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**Increased survival time after tumor resection, but not time to primary tumor resection, correlates with metastatic occurrence.** As luciferase could not be detected in metastatic tumors when transduced WAP-Myc cells were transplanted, we developed a new transgenic luciferase expressing WAP-Myc mouse line (FVB/N-tg(Wap-Myc; CAG-luc,-GFP)) by crossing WAP-Myc mice with FVB/N-tg(CAG-luc,-GFP)
mice. Donor tumors developed with similar kinetics in these mice as in WAP-Myc mice (data not shown). Our preliminary experiment (Fig. 1b) suggested that freezing and thawing cells prior to transplantation correlates with increased metastatic tumor development. Therefore, frozen-and-thawed FVB/N-tg(Wap-Myc; CAG-luc,-GFP) donor tumor cells were syngrafted. In addition, as increased time until tumor resection correlates with increased occurrence of metastasis, we decreased the number of syngrafted cells to 25,000 and 50,000 (eight mice each). Two mice (one in each group) had to be sacrificed during the course of the experiment for reasons unrelated to WAP-Myc tumorigenesis and were eliminated from analysis. Primary tumors were surgically removed when one of the tumors reached 750 mm³, which happened at 5–13 and 5–9 weeks after syngrafting in mice with 25,000 and 50,000 syngrafted cells, respectively (Fig. 2a). At the endpoint, 4 out of 14 mice had devel-
Metastatic tumors occur in 28% of mice with syngrafted WAP-Myc tumors after primary tumor surgery (Fig. 2a). The time frame between fat pad transplantation to primary tumor resection did not affect the chance of metastasis (Fig. 2b). However, prolonged time between tumor resection and sacrifice positively correlated with the likelihood of metastatic growth (Fig. 2c). We could not detect any metastatic tumors through in vivo bioluminescence imaging, despite the facts that lung metastases were fairly large and the primary tumor was detectable (data not shown).

Metastatic tumors maintain the histopathological features of the donor tumor. We next compared the histopathological characteristics of the FVB/N-tg(Wap-Myc; CAG-luc,-GFP) mammary donor tumors to the syngrafted primary tumors and metastatic lung tumors (Fig. 3). Syngrafted primary tumors and metastatic lung tumors retained the strong c-myc expression of donor tumors. Cytokeratin 14, a marker for myoepithelial cells, and collagen IV, a marker for basal lamina, were expressed to similar levels and with similar localization in all tumors. In addition, all tumors were highly proliferative, as evidenced by the expression of Ki67 antigen. Thus, metastatic WAP-Myc tumors recapitulate the morphological characteristics of syngrafted primary tumors, which closely mimic the histopathology of donor tumors.

Metastatic WAP-Myc tumors contain increased numbers of tumor-infiltrating T cells compared to primary tumors. We previously showed that apoptosis in WAP-Myc tumors, induced with venetoclax and metformin treatment, triggered an anti-tumor immune response, and we identified a role for T cells in this response. To gain insight into the immune contexture of WAP-Myc metastatic tumors, we immunoprofiled 6 matching primary and metastatic lung tumors from two independent experiments using flow cytometry analy-
ses. Four tumor pairs were from the experiment with FVB/N-tg(Wap-Myc; CAG-luc,-GFP) mice (Figs. 2 and 3). The other two tumor pairs were from an experiment with the same experimental setup, except that donor tumor cells from WAP-Myc mice (without GFP;Luc) were syngrafted. In the latter experiment, 25% (2 out of 8; time line data not shown) of mice syngrafted with WAP-Myc donor tumor cells formed metastatic tumors, similar as in the experiment with WAP-Myc;GFP;Luc mice (28%; Fig. 2a).

Immunoprofiling data from these tumor pairs are shown in Figs. 4, 5, 6. We observed significantly higher T cell infiltration (both cytotoxic T (CD8+) cells and T helper (CD4+) cells) in metastatic tumors compared to primary tumors from the same mouse, whereas infiltration of other immune cell types did not show significant differences (Fig. 4a,b). Immunohistochemical staining for CD3, a marker of all T cells, confirmed the increased T cell infiltration in metastatic tumors compared to donor and syngrafted primary tumors (Fig. 4c). Immunoprofiling of secondary tumors, which had formed in 3 out of 6 mice with metastasis at the end of the experiment, revealed lower T cell infiltration in secondary tumors compared to metastases (Fig. 5), suggesting that the increased T cell infiltration in metastatic tumors is specific and not due to systemic changes, for example due to mouse development. Staining for T cell activity markers showed that cytotoxic T cells (CD8+) infiltrated in lung metastases expressed more interferon gamma expression compared to cytotoxic T cells in the matching primary tumors (Fig. 6). No other significant changes in T cell activity markers were observed between metastases and primary tumors (Fig. 6).

Discussion
MYC is a potent oncogene that drives tumor initiation and maintenance. As MYC is frequently amplified or overexpressed in high-grade breast tumors25,31, substantial efforts have been put forth to target MYC, either directly21–23 or by exploiting MYC-induced cancer vulnerabilities21,24,25. Therefore, a need exists for preclinical models to study the therapeutic response to MYC targeting in vivo. While recent papers describe spontaneous progression to metastasis in MYC-driven KRASG12D models of lung32 and pancreatic cancer33, to the best of our knowledge, there are no MYC-driven models of breast cancer to represent advanced, metastatic tumors. A few mouse models of breast cancer are driven by MYC, such as WAP-MYC and MMTV-MYC, but they are poorly metastatic27,34,35. In this study, we aimed to generate a clinically-relevant, MYC-driven metastatic breast cancer mouse model by syngrafting WAP-Myc tumor cells to syngeneic recipient mice. To extend the survival of the syngrafted mice and thus increase the window of opportunity for tumor cells to establish metastases, the primary tumor was removed via surgical resection when it reached 750 mm3. This experimental approach has been successfully applied to generate metastatic mouse models using other transgenic mouse lines27,34. We found that survival time from tumor resection to disease progression correlated with likelihood of metastatic spread, which occurred in around 25% of the mice. It is well-established that the microenvironment plays a critical role

Figure 3. Histopathological features are similar in WAP-Myc mammary donor tumors, syngrafted primary tumors and metastatic lung tumors. Representative immunohistochemistry images from FVB/N-tg(Wap-Myc; CAG-luc,-GFP) mammary donor tumors, syngrafted primary tumors and metastatic lung tumors, stained as indicated. Scale bar = 50 μm.
Figure 4. Metastatic lung tumors have higher T-cell infiltration compared to primary tumors. (a) Flow cytometry analyses of the number of leukocytes (CD45+), total T cells (CD3+), T helper cells (CD3+CD4+), cytotoxic T cells (CD3+CD8+), NK cells (CD3−NK1.1+), NKT cells (CD3+NK1.1+), monocytes (CD11b+Ly6C+) and neutrophils (CD11b+Ly6G+) in 6 matching primary and metastatic lung tumors. Four tumor pairs were from mice transplanted with WAP-Myc;GFP;Luc cells (as in Figs. 2, 3) and two tumor pairs were from mice transplanted with WAP-Myc cells (indicated in green and black, respectively). A paired t-test was used to compare primary and metastatic lung tumors (* p < 0.05; CD3+ cells, p = 0.0154; CD4+ cells p = 0.0235). (b) Flow cytometry gating strategies for the immune cell populations quantified in (a). Gating examples are from the primary tumor from the same mouse. (c) Representative immunohistochemistry images of donor tumor slices from two mice and matched syngrafted primary tumor and lung metastasis stained for T cell marker CD3. Scale bar = 50 μm.
in metastatic processes\textsuperscript{36}, and it should be pointed out that our syngrafting experiments were performed with heterogeneous tumor material including tumor cells, mesenchymal and residual fat cells, and immune cells. For example, a study by Ricke and coworkers\textsuperscript{37} showed that while small non-invasive tumors developed when immortalized prostatic epithelial cells were transplanted alone, tumors with metastatic ability were generated only after transplantation of mice with mesenchymal cells and immortalized prostatic epithelial cells. Therefore, it is highly likely that at least part of the metastatic capacity in syngrafted tumors involves the stromal component of the tumor syngrafts. Given that injection of tumor cells into tail veins of mice commonly leads to lung metastases, we cannot fully exclude the possibility that some metastases were contributed by direct leakage of transplanted cells into the bloodstream through microvessels. This is unlikely, however, given that transplantation of 100,000 fresh cells (Fig. 1) gave rise to fewer metastases than transplantation of 25,000 or 50,000 fresh cells (Fig. 2) and injecting breast cancer cells in the mouse mammary fat-pad is a relatively simple procedure unlikely to result in significant tumor cell leakage\textsuperscript{38,39}.

The overall metastasis rate of 25\% is unfavorable to reliably assess the effects of different drugs on metastasis, as very large numbers animals would need to be used in one experiment. For example, in order to reliably obtain eight mice with metastasis, more than 32 mice have to be transplanted per treatment group, thus requiring large quantities of mice and drugs. However, the WAP-Myc metastatic breast cancer model described here can be a valuable tool to study the mechanism of metastasis in aggressive MYC-high breast tumors. The advantage of the WAP-Myc model is that it maintains all the crucial biological steps of the metastatic process and closely mimics the clinical progression of metastatic breast cancer in patients. To increase the metastatic frequency, tumor cells from these spontaneous metastatic tumors can be passaged in vivo through serial transplantations to select highly metastatic variants\textsuperscript{40,41}. However, these variants may lack the heterogeneity of the transplanted donor tumors. Another approach to render the WAP-Myc tumor cells more metastatic is to introduce additional mutations or changes in the expression of candidate genes. For example, a previous study showed that overexpression of the pro-angiogenic factor VEGF in the MMTV-MYC mammary tumor model resulted in much higher pulmonary metastasis rates\textsuperscript{42}.

We have previously used the WAP-Myc syngraft model to demonstrate a new therapeutic strategy for MYC-high breast cancer\textsuperscript{25}. A combination of the Bcl-2 inhibitor venetoclax and anti-diabetic drug metformin induced cell death specifically in MYC-high tumors, and this response was augmented by checkpoint inhibitor anti-PD-1, which prevents dampening the T-cell-mediated immune response through PD-1/PD-L1 (Programmed death-ligand 1) interaction. In our current study, we found increased infiltration of T cell populations in the metastatic tumors compared to the primary tumors (Fig. 4a,b), while other immune cell types showed similar levels of infiltration. In addition, cytotoxic T cells infiltrating in WAP-Myc metastases express higher levels of interferon gamma compared to primary cells (Fig. 4c). Interferon gamma can play multiple roles in cancer, both pro- and anti-tumorogenic, from increasing cytotoxicity and motility\textsuperscript{43} to downregulation of major histocompatibility

\textbf{Figure 5.} Metastatic lung tumors have higher T-cell infiltration compared to secondary tumors. (a) Flow cytometry analyses as in Fig. 4a in 3 matching primary, secondary and metastatic lung tumors. One tumor pair was from mice transplanted with WAP-Myc;GFP;Luc cells and two tumor pairs were from mice transplanted with WAP-Myc cells, indicated in green and black, respectively.

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\caption{Metastatic lung tumors have higher T-cell infiltration compared to secondary tumors. (a) Flow cytometry analyses as in Fig. 4a in 3 matching primary, secondary and metastatic lung tumors. One tumor pair was from mice transplanted with WAP-Myc;GFP;Luc cells and two tumor pairs were from mice transplanted with WAP-Myc cells, indicated in green and black, respectively.}
\end{figure}
complexes and upregulation of checkpoint inhibitors such as PD-L1\(^44\). These data could suggest that MYC-driven lung metastasis might benefit from treatments that reinvigorate the immune system, for example with a drug combo that combines induction of cancer cell killing and immune checkpoint blockade such as the aforementioned combination of venetoclax, metformin and anti-PD-1\(^25\). Further studies will be needed to test this hypothesis.

Classically, T cells have been thought as tumor suppressive, however, certain subtypes of T helper cells, such as regulatory T cells or pro-inflammatory IL-17-expressing Th17 cells, have immune suppressive functions in tumors and promote metastasis to the lungs\(^45\). As the immunoprofiling panels used in this study do not cover all immune cell types, it is important to characterize the T cell infiltrates and cytokine profiles in metastatic tumors and determine their function in prospective studies.

Figure 6. Higher interferon gamma expression in cytotoxic T cells (CD8\(^+\)) in lung metastases compared to matching primary tumors. (a) Flow cytometry analyses of PD-1, CD107a, Granzyme B and interferon gamma expression in T helper cells (CD3\(^+\) CD4\(^+\)) and cytotoxic T cells (CD3\(^+\) CD8\(^+\)) in the same 6 matching primary and metastatic lung tumors as analysed in Fig. 4a. Four tumor pairs were from mice transplanted with WAP-Myc;GFP;Luc cells (as in Figs. 2, 3) and two tumor pairs were from mice transplanted with WAP-Myc cells (indicated in green and black, respectively). A paired t-test was used to compare primary and metastatic lung tumors (* p = 0.0494). (b) Flow cytometry gating strategies for the markers quantified in (a) as exemplified using cytotoxic T cells (CD8\(^+\)) from metastases and primary tumor samples from the same mouse.
In summary, we have developed a robust WAP-Myc mammary tumor transplant mouse model that shows moderate spontaneous autochthonous metastasis to lungs. This model can be used to study the mechanisms of metastasis in MYC-driven breast cancer in future studies.

Data availability
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions
B.U., J.P. and J.K. designed the study. B.U., J.L. and R.T. performed the experiments and analyzed the data. B.U., R.T., J.L. and J.P. and J.K. wrote the manuscript. R.T. and J.L. equally contributed as second authors. J.P. and J.K. equally contributed as senior and corresponding authors.

Competing interests
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Additional information
Correspondence and requests for materials should be addressed to J.P. or J.K.

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