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SHORT COMMUNICATION

The Rap GTPases regulate the migration, invasiveness and *in vivo* dissemination of B-cell lymphomas

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B-cell lymphomas are common malignancies in which transformed B cells enter the circulation, extravasate into tissues and form tumors in multiple organs. Lymphoma cells are thought to exit the vasculature and enter tissues through the same chemokine- and adhesion molecule-dependent mechanisms as normal B cells. We have previously shown that activation of the Rap GTPases, proteins that control cytoskeletal organization and integrin activation, is critical for chemokine-induced migration and adhesion in B-lymphoma cell lines. Using the A20 murine B-lymphoma cell line as a model, we now show that Rap activation is important for circulating lymphoma cells to enter tissues and form tumors *in vivo*. *In vitro* assays showed that Rap activation is required for A20 cells to efficiently adhere to vascular endothelial cells and undergo transendothelial migration. These findings suggest that Rap or its effectors could be novel targets for treating B-cell lymphomas.
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The ability of normal and malignant B cells to leave the circulation and enter tissues depends on homeostatic chemokines, such as CXCL12, CCL19, CCL21 and CXCL13, which are immobilized on the surface of vascular endothelial cells (Pals *et al*., 2007; Thelen and Stein, 2008). Chemokine receptor signaling activates the lymphocyte integrins LFA-1 and VLA-4, permitting firm adhesion to the endothelial cells. This leads to chemokine-induced migration of the lymphocyte along the surface of the endothelial cells, followed by transendothelial migration into the underlying tissue. Lymphoma cells that can establish a suitable metastatic niche may then form tumors.

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The Rap GTPases (Rap1a, Rap1b, Rap2a, Rap2b and Rap2c; collectively referred to as Rap) are signal-transducing switches that cycle between an active GTP-bound form and an inactive GDP-bound form. Activated Rap is a master regulator of cytoskeletal organization and integrin activation (Bos, 2005). In lymphocytes, chemokine receptor signaling leads to Rap activation and this is critical for chemokines to stimulate cell migration and integrin-mediated adhesion (McLeod *et al*., 2002, 2004; Shimonaka *et al*., 2003; Durand *et al*., 2006). However, the role of Rap activation in the *in vivo* dissemination and establishment of B-cell lymphomas is not known. Therefore, we asked whether blocking Rap activation in the A20 murine B-lymphoma cell line (Kim *et al*., 1979) could reduce the ability of these cells to form tumors when injected intravenously (i.v.) into Balb/c mice, a well-defined syngeneic model of B-cell lymphoma (Staveley-O'Carroll *et al*., 1998). A20 cells are a relevant model system as they resemble human diffuse large B-cell lymphomas, which account for 40% of non-Hodgkin's lymphomas.

To block Rap activation, we transduced A20 cells with a bicistronic vector designed to co-express green fluorescent protein (GFP) and RapGAPII (Figure 1a), a Rap-specific GTPase-activating protein (GAP) that converts Rap1 and Rap2 to their inactive GDP-bound forms. Expressing RapGAPII in A20 cells completely blocked activation of Rap1 and Rap2 by CXCL12 (Figure 1b), a chemokine that is produced in the lymphoid organs, bone marrow, liver, lungs and ovaries (Shirozu *et al*., 1995) and which has a key role in the dissemination of many types of tumors. RapGAPII expression selectively blocks CXCL12-induced Rap activation and does not affect CXCL12-induced activation of Akt/protein kinase B, MAP kinases or the Rac1 GTPase in B-lymphoma cell lines (McLeod *et al*., 2002; Durand *et al*., 2006). Moreover, expressing RapGAPII in A20 cells did not alter the levels of Rap1 or Rap2 (Figure 1b), did not reduce cell surface expression of CXCR4 (the receptor for CXCL12), LFA-1 or VLA-4 (Figure 1c), and did not alter the *in vitro* proliferation of A20 cells (Figure 1d).

To test whether Rap activation is important for the *in vivo* dissemination and establishment of B-cell lymphomas, GFP-expressing A20/vector cells or A20/RapGAPII cells were injected i.v. into Balb/c mice. Mice were monitored daily for signs of tumor progression

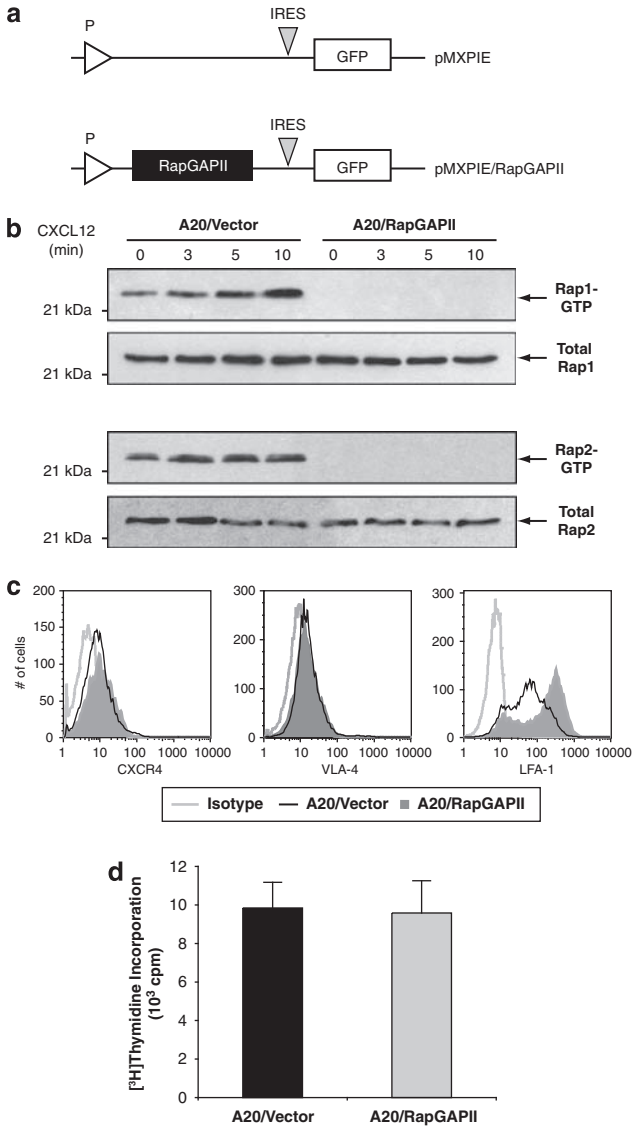

Figure 1 RapGAPII expression blocks CXCL12-induced activation of Rap1 and Rap2 in A20 cells. (**a**) Schematic of the green fluorescent protein (GFP)-encoding retroviral vector pMXPIE and the pMXPIE/RapGAPII vector, which encodes RapGAPII and GFP on a single transcript separated by an internal ribosome entry site (IRES). P represents the retroviral promoter. Bulk populations of A20 cells stably expressing either pMXPIE or pMXPIE/RapGAPII were generated by retroviral transduction, as described (Krebs *et al*., 1999). (**b**) A20/vector and A20/RapGAPII cells (5×10^6) were stimulated with 100 ng/ml CXCL12 (R&D Systems, Minneapolis, MN, USA) for the indicated times. The upper panels show the active GTP-bound forms of Rap1 and Rap2, which were precipitated using a glutathione *S*-transferase–RalGDS fusion protein and visualized by immunoblotting, as described (McLeod *et al*., 1998). The lower panels show total Rap1 and Rap2 in the cell lysates. (**c**) Fluorescence-activated cell sorting analysis of A20/vector and A20/RapGAPII cells stained with monoclonal antibodies to CXCR4 (clone 2B11; eBioscience, San Diego, CA, USA), VLA-4 (clone R1-2; eBioscience) or LFA-1 (clone M17/4; eBioscience), as well as isotype-matched control antibodies. (**d**) Cell proliferation was assessed by culturing cells (2×10^4 per well of a 96-well plate) for 48 h before adding 1 μCi of [^{3}H]thymidine per well and measuring [^{3}H]thymidine incorporation into DNA (mean ± s.d. for triplicate wells) 4 h later. For (**b**–**d**), similar results were obtained in three experiments.

(abdominal swelling, scruffy fur, decreased movement, weight loss and hunched posture), at which point they were euthanized and examined for tumors. When injected with A20/vector cells, 50% of the mice exhibited these symptoms after 31 days and by day 43 all of the mice had to be euthanized (Figure 2a). Preventing Rap activation significantly delayed and reduced the incidence of tumor formation. When the mice were injected with A20/RapGAPII cells, 50% of the mice did not require euthanization until day 49, 18 days later than mice injected with A20/vector cells. Moreover, 30% of the mice injected with A20/RapGAPII cells did not develop any signs of tumor formation up to 90 days after injection (Figure 2a).

In mice that developed tumors, lymphomas occurred in multiple organs, including the liver, ovaries, lymph nodes, peritoneal cavity, spleen and bone marrow. In general, A20/vector cells established tumors at more sites than A20/RapGAPII cells. The liver, which produces large amounts of CXCL12 (Goddard *et al*., 2001), was the preferential site of lymphoma development. All mice that developed tumors first exhibited tumors in the liver, before developing visible tumors at other sites. When we examined the livers of mice at 3 and 4 weeks after i.v. injection with A20 cells, fewer mice injected with A20/RapGAPII cells had detectable liver tumors than mice injected with A20/vector cells (Figure 2b). In addition, mice injected with A20/vector cells usually exhibited 5–7 distinct tumor nodules in the liver, whereas the mice that developed tumors after being injected with A20/RapGAPII cells had only 1–2 tumor nodules (Figure 2c). Thus, blocking Rap activation reduced lymphoma dissemination *in vivo*.

A closer examination of tumors isolated from mice injected with A20/RapGAPII cells showed that all the tumor cells had reduced levels of both GFP and the FLAG-RapGAPII protein, compared with the parental cell A20/RapGAPII cells that had been kept in culture for 3 months (Figures 2d and e). Cells derived from these tumors had lost expression of the 100-kDa FLAG-RapGAPII protein to varying degrees, with some (for example, A20/RapGAPII tumors #1, 3 and 8) having barely detectable levels of FLAG-RapGAPII (Figure 2e). Loss of the 100-kDa FLAG-RapGAPII band was often accompanied by increased levels of a lower molecular weight anti-FLAG-reactive band that is just barely detectable in the *in vitro*-cultured A20/RapGAPII cells. It is not clear whether this is a functional isoform of RapGAPII. Nevertheless, there appears to have been an *in vivo* selection for cells with reduced expression of FLAG-RapGAPII, perhaps reflecting a critical role for Rap activation in lymphoma dissemination or tumor formation.

Rap activation assays showed that cells from A20/RapGAPII tumor #1, which had barely detectable levels of the 100-kDa FLAG-RapGAPII, were able to activate Rap in response to CXCL12 to the same degree as A20/vector cells (Figure 2f). Cells derived from A20/RapGAPII tumor #3, which had low levels of FLAG-RapGAPII, were also capable of activating Rap in response to CXCL12 (Figure 2f). Although Rap1 acti-

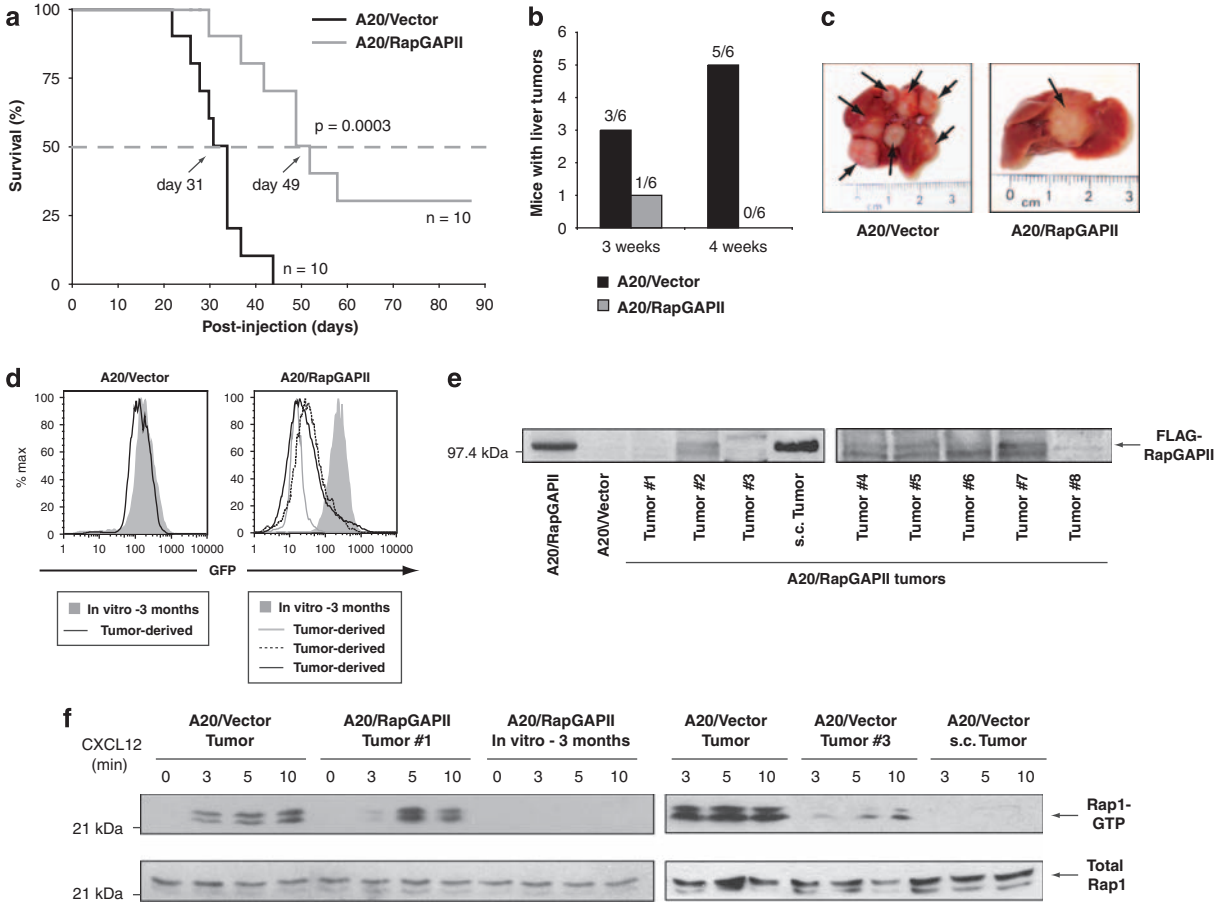

Figure 2 Blocking Rap activation reduces lymphoma dissemination and development *in vivo*. (**a**) Kaplan–Meier survival curves for groups of ten 6–8 weeks old Balb/c mice that received 5×10^6 green fluorescent protein (GFP)-expressing A20/vector or A20/RapGAPII cells intravenously (i.v.) through injection into the tail vein. The mice were euthanized when they exhibited signs of tumor development, such as abdominal swelling, scruffy fur, decreased movement, weight loss or hunched posture, in accord with the University of British Columbia Animal Care Committee policies. The data are combined from two independent experiments. $P = 0.0003$ using the Mantel–Cox log–rank test. (**b**) Groups of six Balb/c mice were injected with either A20/vector or A20/RapGAPII cells and the presence of visible liver tumors was assessed after 3 or 4 weeks. (**c**) Images of the liver lobes from mice that developed tumors after being injected i.v. with A20/vector or A20/RapGAPII cells. The arrows point to distinct tumor nodules. (**d**) Representative fluorescence-activated cell sorting (FACS) plots of GFP expression for cells kept in culture versus cells isolated from tumors. FACS traces are shown for three tumors isolated from different mice that had been injected with A20/RapGAPII cells. (**e**) FLAG-RapGAPII expression in cell extracts (30 μg protein) from cells isolated from eight different tumors that developed in mice injected with A20/RapGAPII cells, as well as from A20/RapGAPII cells and A20/vector cells that were cultured *in vitro* for 3 months, was assessed by immunoblotting with the M2 anti-FLAG monoclonal antibody (Sigma-Aldrich, St Louis, MO, USA). Note that cells isolated from subcutaneous (s.c.) tumors that developed after s.c. injection of A20/RapGAPII cells maintained high expression of FLAG-RapGAPII, unlike cells isolated from tumors that developed after i.v. injection of A20/RapGAPII cells. (**f**) Cells isolated from tumors arising after i.v. injection (A20/vector tumor, A20/RapGAPII tumors #1 and #3) or s.c. injection, as well as parental A20/RapGAPII and A20/vector cells that were maintained in cultured for 3 months, were stimulated with 100 ng/ml CXCL12 and assayed for Rap1 activation as in Figure 1.

vation in these cells was only ~20% of that in A20/vector cells, this was apparently sufficient to allow tumor formation. As RapGAPII inactivates Rap GTPases in an enzymatic manner, the magnitude of CXCL12-induced Rap activation would be expected to be even lower in other A20/RapGAPII tumors with higher levels of FLAG-RapGAPII (for example, A20/RapGAPII tumors #4–#7). This suggests that quite low levels of Rap activation may be sufficient for A20 lymphoma cells to form liver tumors after i.v. injection. However, the *in vivo* dissemination and development of B-cell lymphomas seems to require some level of Rap activation. No A20/RapGAPII-derived tumors were recovered that exhibited the high level of FLAG-RapGAPII expression present in the pre-injection A20/RapGAPII cell population. This may reflect an *in vivo* selection that favored a minor fraction of the original A20/RapGAPII cell population that had reduced expression of the bicistronic mRNA-encoding GFP and RapGAPII, and which were therefore able to activate the Rap GTPases to some extent.

The failure of A20 cells expressing high levels of FLAG-RapGAPII to form tumors after i.v. injection could reflect an inability of these cells to survive *in vivo* or their inability to exit the vasculature and migrate to a niche suitable for tumor growth. We ruled out the first

possibility by showing that A20/RapGAPII did form tumors when 5×10^5 cells were injected subcutaneously into the flanks of Balb/c mice. Cells derived from these subcutaneous tumors continued to express RapGAPII (Figure 2e) and exhibited complete inhibition of Rap activation (Figure 2f). Thus, Rap activation is not required for A20 cells to survive *in vivo*. Therefore, we investigated whether the ability of circulating B-cell lymphomas to cross the endothelial cell layers and exit the vasculature was dependent on Rap activation.

Using an *in vitro* model of extravasation, we found that blocking Rap activation reduced CXCL12-dependent transmigration of A20 cells through a confluent monolayer of bEND.3 mouse microvascular endothelial cells by >50% (Figure 3a). This reduced transmigration capacity of *in vitro*-cultured A20/RapGAPII cells, which have undetectable amounts of Rap activation, reflected deficits in multiple aspects of the transmigration process. Adhesion to vascular endothelial cells is a prerequisite for lymphocyte extravasation and we found that A20/RapGAPII cells exhibited a markedly reduced ability to adhere to bEND.3 cells, compared with A20/vector cells (Figure 3b). Once lymphocytes have adhered to vascular endothelial cells, they move across the surface of endothelial cells until they find a suitable site for extravasation. This motility is mediated by leukocyte integrins that bind the adhesion molecules on the vascular endothelial cells and facilitated by chemokines that are immobilized on the surface of the endothelial cells. A20/RapGAPII cells exhibited a significantly reduced ability to migrate across fibronectin-coated Transwells (Corning, Lowell, MA, USA) in response to CXCL12 (Figure 3c), suggesting that their integrin-dependent motility is reduced compared with A20/vector cells. Finally, once lymphocytes contact the junctions between endothelial cells, they must extend membrane processes into these junctions in order to undergo transmigration through the endothelial cell layer. Confocal imaging revealed that ~10% of A20/vector cells that had adhered to bEND.3 cells extended membrane processes between and underneath these endothelial cells (Figure 3d). Of the much smaller number of A20/RapGAPII cells that adhered to bEND.3 cells, the fraction that extended invadopodia-like membrane processes beneath the endothelial cells was 40% lower than that for the A20/vector cells (Figure 3d, right panel). This may reflect a decreased ability of the A20/RapGAPII cells to move to junctions between endothelial cells, as well as a decreased ability to undergo the cytoskeletal reorganization involved in the formation of invadopodia. Thus, at least *in vitro*, Rap activation facilitates the chemokine-induced transendothelial migration of B-lymphoma cells by promoting multiple steps in this process.

To determine whether the decreased ability of A20/RapGAPII cells to cross endothelial cell layers and migrate on extracellular matrix components *in vitro* correlated with a decreased ability to exit the vasculature and invade organs *in vivo*, equal numbers of A20/vector cells and A20/RapGAPII cells that had been differentially labeled with CellTracker (Invitrogen, Burlington, Ontario, Canada) dyes were co-injected into mice. After 24 h, the relative number of A20/vector cells and A20/RapGAPII cells that had lodged in the liver was determined. Confocal microscopy of liver sections showed that A20/vector cells readily invaded the liver over a 24-h period, whereas A20/RapGAPII cells did so at a lower frequency (Figures 4a and b). Although injected in equal numbers, at both 24 and 72 h after injection, ~75% of the cells lodging in the liver were A20/vector cells, whereas only 25% were A20/RapGAPII cells (Figure 4b).

The A20/vector cells that lodged in the liver frequently (~60%) assumed a 'spread' ameboid morphology that is characteristic of extravasating and migrating cells (Figures 4c and d, left panel). Their intimate association with hepatocytes suggested that the majority (60–70%) of the A20/vector cells had lodged in the liver parenchyma at 24 and 72 h after injection (Figures 4c and d, center panel). The remainder of the A20/vector cells appeared to be in unstained interstitial spaces, possibly the liver sinusoids, and may represent cells that were not flushed out of the vasculature by the perfusion process. Approximately 50% of the A20/vector cells that had lodged in the liver exhibited an ameboid morphology and were clearly within the liver parenchyma (Figure 4d, right panel). The combination of these two criteria likely reflects cells that are the most invasive and have the greatest potential to form tumors.

A20/RapGAPII cells lodged in the liver at about one-third the efficiency of A20/vector cells (Figure 4b), and of these cells, fewer developed a spread morphology and infiltrated the liver parenchyma than was the case for A20/vector cells (Figures 4c and d). Importantly, the percentage of A20/RapGAPII cells that were both spread and within the liver parenchyma was significantly lower than for A20/vector cells (20 versus 50%; Figure 4d, right panel). A larger fraction of the A20/RapGAPII cells were round and in the interstitial spaces, as opposed to being in contact with hepatocytes (Figures 4c and d). Thus, Rap activation is important for A20 cells to efficiently infiltrate organs, such as the liver, and lodge in sites that may be suitable microenvironments for tumor growth.

In this report, we have shown for the first time that activation of the Rap GTPases is critical for the *in vivo* dissemination of B-cell lymphomas. In particular, we found that blocking Rap activation substantially reduced the ability of circulating lymphoma cells to exit the vasculature *in vivo* and enter the liver parenchyma, a site where these cells readily form tumors. This finding is consistent with previous study showing that the ability of leukemic blast cells to enter organs in mice can be reduced by expressing SPA-1 (Ishida *et al*., 2003), another Rap-specific GAP that inactivates Rap. The role of Rap activation in the extravasation of lymphoma cells likely reflects its critical function in cell adhesion and cell migration. We had previously shown that Rap activation is required for A20 cells to undergo α4-integrin-mediated adhesion to bone marrow stromal cells (McLeod *et al*., 2004). We have now extended these findings to show that Rap activation is important for A20 B-lymphoma cells to adhere to vascular endothelial cells, migrate on adhesion molecule-coated surfaces,

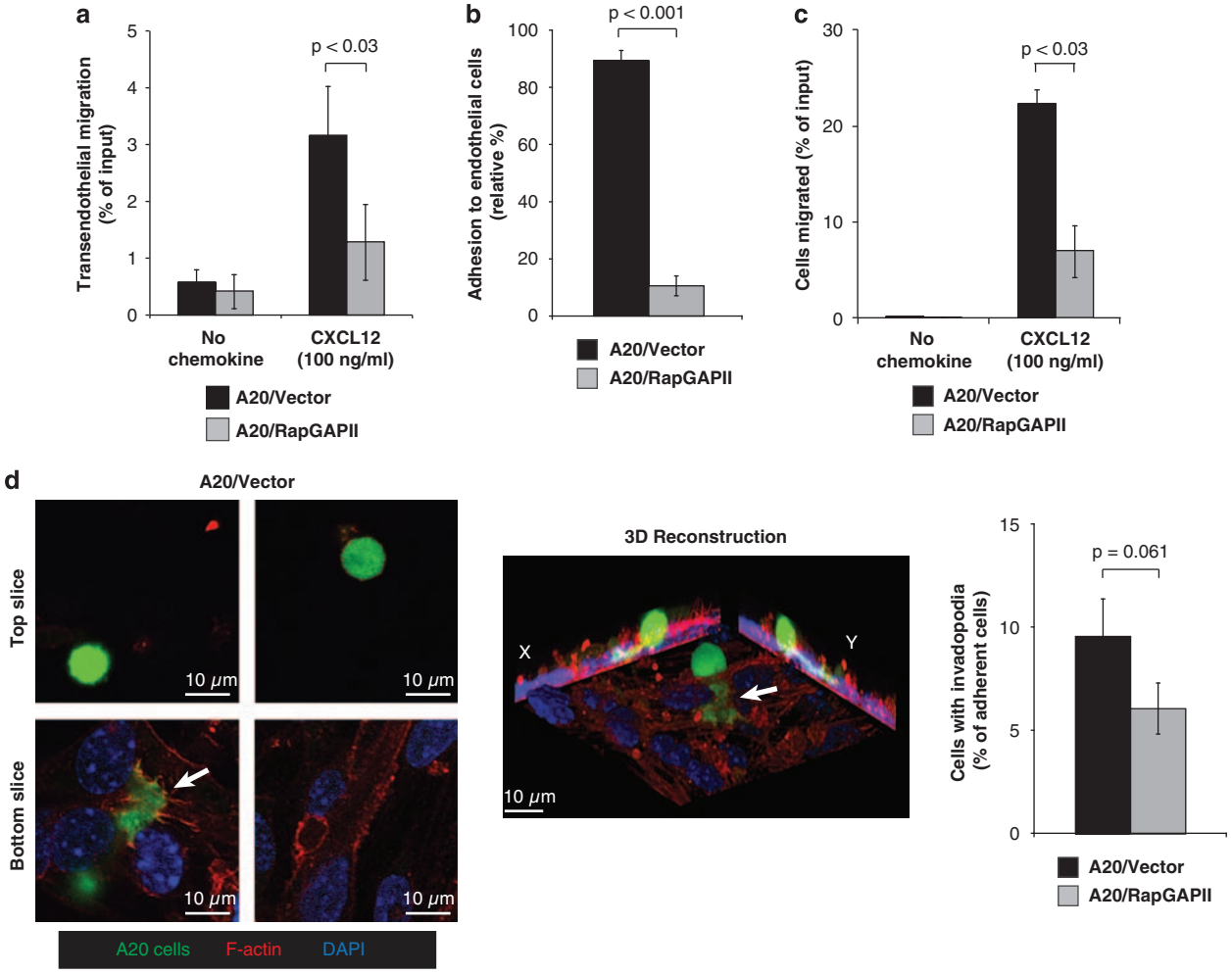

Figure 3 Rap activation is important for transendothelial migration, adhesion to vascular endothelial cells and integrin-mediated migration by A20 cells. (**a**) Transendothelial migration was assessed using clear tissue culture well inserts (8-μm pores; BD Biosciences, Mississauga, Ontario, Canada) that were coated with 2 μg/ml fibronectin (R&D Systems) and then seeded with bEnd.3 murine microvasculature endothelial cells (Sikorski *et al*., 1993). The bEND.3 cells were grown to confluence and activated overnight with 10 ng/ml tumor-necrosis factor-α (TNF-α) (eBioscience) before adding 100 ng/ml CXCL12 to the lower chamber and A20/vector or A20/RapGAPII cells to the upper chamber. The percentage of A20 cells that migrated into the lower chamber after 16 h (mean ± s.e.m. for four experiments) is shown. (**b**) The relative ability of A20/vector and A20/RapGAPII cells to adhere to vascular endothelial cells was assessed. bEnd.3 cells were cultured overnight with 10 ng/ml TNF-α in 8-well μ-Slides (ibidi, Martinsried, Germany) that had been coated with 1 μg/ml fibronectin A20/vector, and A20/RapGAPII cells were then labeled with different CellTracker dyes (Invitrogen), mixed in equal numbers, and allowed to adhere to the bEnd.3 cells for 16 h. Non-adherent cells were removed before fixing the cells with 4% paraformaldehyde and staining nuclei with 4,6-diamidino-2-phenylindole (DAPI). Images were captured using an Olympus IX81/Fluoview FV1000 confocal microscope (Olympus, Markham, Ontario, Canada). The relative numbers of A20/vector and A20/RapGAPII cells that adhered to the bEND.3 cells is expressed as a percentage of the total (100%). The mean ± s.e.m. is shown for four experiments, two experiments in which the A20/vector cells were labeled with CellTracker Green and the A20/RapGAPII cells with Cell Tracker Orange, and two in which the dyes were reversed. (**c**) The migration of A20/vector and A20/RapGAPII cells towards 100 ng/ml CXCL12 was assessed as described (McLeod *et al*., 2002) using Transwell filters (5-μm pores; Costar) coated with 2 μg/ml fibronectin. After 5 h, the percentage of input cells that had migrated into the lower chamber was determined. The mean ± s.e.m. for four experiments is shown. (**d**) To assess the formation of invadopodia, A20/vector or A20/RapGAPII cells were labeled with CellTracker Green and added to confluent monolayers of TNF-α-activated bEnd.3 cells, as in panel **b**. After 16 h, cells were fixed, permeabilized with 0.5% Tween, and stained with DAPI plus rhodamine–phalloidin to detect F-actin. The left panel shows representative confocal images of the top (above the endothelial cells) and bottom (closest to the substrate, that is, below the endothelial cells) slices from *z* axis stacks for A20/vector cells interacting with bEND.3 cells. The pair of slices on the left shows an A20/vector cell (green) that had invaded between two endothelial cells (indicated by the red F-actin staining and the large blue nucleus) and contacted the substrate. The arrow indicates the membrane process that contacted the substrate. The pair of slices on the right illustrates an A20/vector cell that adhered to the top of the endothelial cells but did not form a membrane process that crossed the endothelial cell layer. The center panel is a three-dimensional reconstruction showing an A20/vector cell that had extended a membrane process under the endothelial cells (arrow), as well as two cells that did not extend invadopodia. The cell indicated by the arrow is the same one shown in the left panel. Similar imaging was performed for A20/RapGAPII cells, of which far fewer adhered to the bEND.3 cells. The right panel shows the percentage of adherent A20/vector and A20/RapGAPII cells that extended invadopodia beneath the bEND.3 cells. The mean ± s.e.m is shown for four experiments in which >300 cells were analysed per experiment. For all panels, *P*-values were determined using Student's paired two-tailed *t*-test.

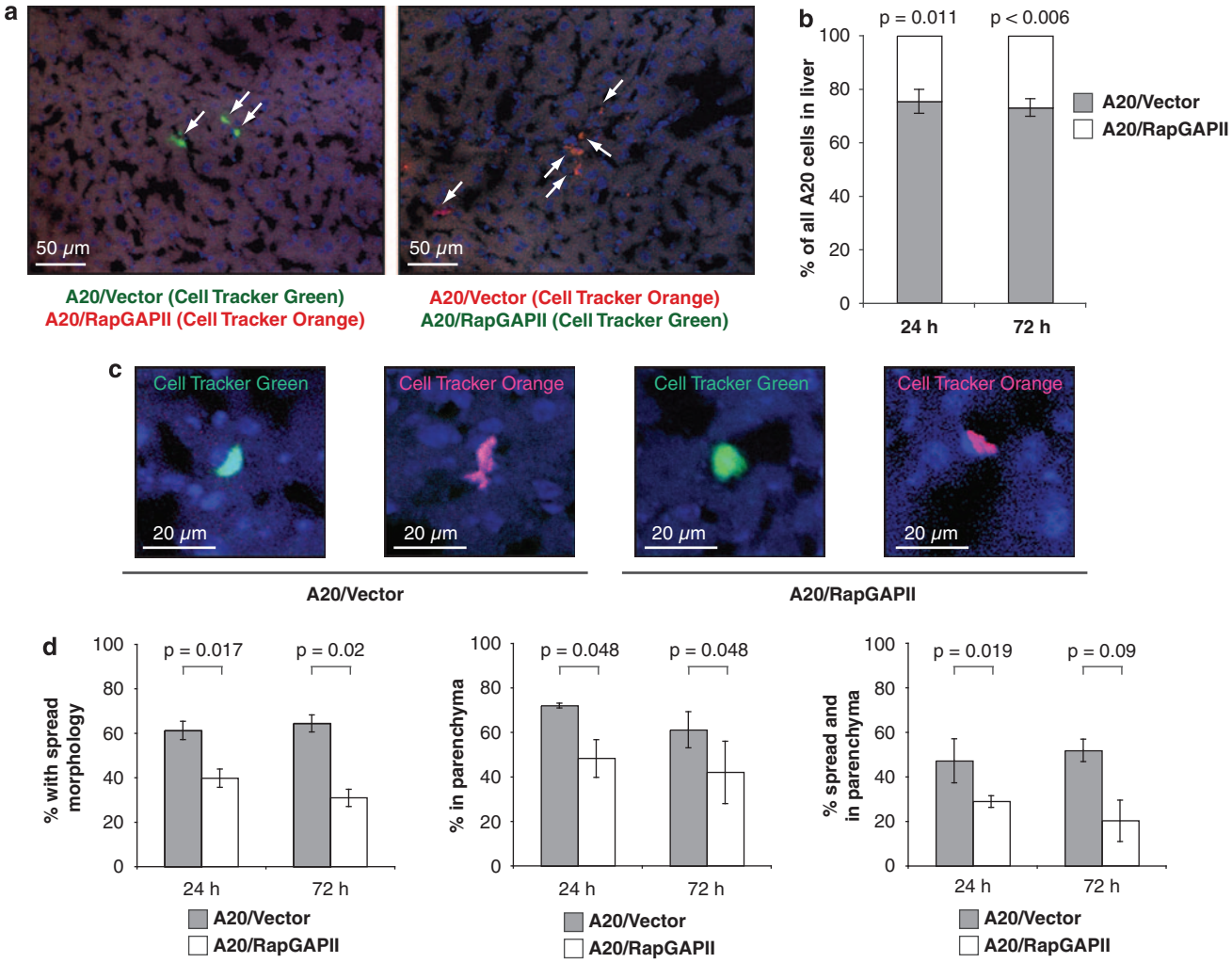

Figure 4 Blocking Rap activation impairs the ability of A20 B-lymphoma cells to invade the liver. A20/vector and A20/RapGAPII cells were differentially labeled with CellTracker Green and CellTracker Orange. Five million cells of each type were mixed together and injected into each of four Balb/c mice. After 24 or 72 h, the mice were euthanized, perfused with phosphate-buffered saline to wash cells out of the vasculature, and liver sections were prepared. Liver samples were embedded in OCT (Fisher Scientific, Ottawa, Ontario, Canada), frozen on dry ice, cut into 7-μm sections using a Cryotome (Thermo Scientific, Waltham, MA, USA), fixed in 4% paraformaldehyde and mounted onto slides with 4,6-diamidino-2-phenylindole (DAPI)-Prolong Gold (Invitrogen). (**a**) Confocal images of liver sections. Arrows indicate A20 cells. DAPI-stained hepatocytes appear blue/purple. (**b**) Quantitative analysis of the relative numbers of A20/vector and A20/RapGAPII cells in the liver at 24 and 72 h after injection. For each mouse injected, a total of 40 cells from multiple sections were counted and the relative numbers of A20/vector and A20/RapGAPII cells are expressed as a percentage of the total (100%). The mean ± s.e.m. is shown for four experiments, two experiments in which the A20/vector cells were labeled with CellTracker Green and the A20/RapGAPII cells with Cell Tracker Orange, and two in which the dye labeling was reversed. (**c**) Higher magnification images showing that some cells that had entered the liver exhibited a spread, ameboid morphology and were localized within the liver parenchyma (that is, were in close contact with hepatocytes, which were identified by DAPI staining (blue) and their intrinsic autofluorescense (purple)). Other cells were round and appeared to be in interstitial spaces within the liver. (**d**) The percentage of A20/vector and A20/RapGAPII cells in the liver at 24 and 72 h after intravenous injection that exhibited a spread morphology (left panel) were localized in the liver parenchyma (center panel), or were both spread and within the liver parenchyma (right panel) was determined for the cells counted in panel **b**. The mean ± s.e.m. is shown for the four experiments, two experiments in which the A20/vector cells were labeled with CellTracker Green and the A20/RapGAPII cells with Cell Tracker Orange, and two in which the dye labeling was reversed. For each parameter, *P*-values for A20/vector versus A20/RapGAPII cells were calculated using paired Student's *t*-test.

extend membrane processes into the junctions between endothelial cells and undergo transendothelial migration *in vitro*. The *in vitro* migration of T cells and chronic lymphocytic leukemia cells across the endothelial monolayers also requires Rap activation (Shimonaka *et al*., 2003; Till *et al*., 2008), suggesting that this is a general requirement for the extravasation of lymphoma cells into the organs.

In addition to being critical regulators of integrin activation and integrin-mediated adhesion, the Rap GTPases are master regulators of actin cytoskeleton organization (Bos, 2005). We have previously shown that Rap activation is important for A20 cells to reorganize their actin cytoskeleton, undergo cell spreading, and form immune synapses in response to B-cell receptor engagement (Lin *et al*., 2008). Activated Rap

regulates the activity and subcellular localization of multiple proteins that control actin dynamics and cell polarity, including the Rac GTPase activators Vav2 and TIAM1, profilin, AF-6, myosin II and the Par3/Par6 polarity complex (Bos, 2005; Gerard *et al*., 2007; Jeon *et al*., 2007). The data presented here support the idea that Rap-dependent cytoskeletal reorganization is important for B-cell lymphomas to move across endothelial cell monolayers, extend membrane processes into junctions between endothelial cells and then move their cell body through these junctions.

Our competitive *in vivo* homing assays showed that A20/RapGAPII cells lodged in the liver with about one-third the efficiency of A20/vector cells and that these cells were half as likely to be within the liver parenchyma. Combining these two factors suggests that A20/RapGAPII cells were about one-sixth as likely as A20/vector cells to exit the vasculature and enter the liver parenchyma. An impaired ability to seed the liver parenchyma likely accounts for the delayed and reduced incidence of liver tumor formation exhibited by the A20/RapGAPII cells.

The analysis of liver tumors that formed after the injection of A20/RapGAPII cells showed that cells from these tumors all had reduced levels of the exogenous FLAG-RapGAPII protein, compared with the pre-injection A20/RapGAPII cell population. Moreover, some of these tumor-derived cells were capable of activating Rap1 to a significant extent. Our finding that Rap activation was required for A20 cells to efficiently adhere to vascular endothelial cells and undergo trans-endothelial migration suggests that these steps could impose an *in vivo* selection in which only those A20/RapGAPII cells with reduced levels of FLAG-RapGAPII expression, and at least a minimal level of Rap activation, are competent to extravasate into the liver parenchyma.

Once malignant cells enter an organ, their ability to form a tumor depends on the interactions with surrounding stromal cells (Chambers *et al*., 2002). Our observation that A20/RapGAPII cells formed subcutaneous tumors while maintaining high levels of FLAG-RapGAPII expression that completely suppress Rap activation suggests that activated Rap is not required for this step. However, adhesive interactions with stromal cells, which are dependent on Rap activation (McLeod *et al*., 2004), may be more important for isolated A20 cells that have invaded tissues than for A20 cells that have been injected into a tissue in large numbers. Although we did not see a preferential loss of A20/RapGAPII cells between the 24 and 72 h time points in our competitive *in vivo* homing assays (Figure 4b), we do not know whether these cells with reduced Rap activation underwent more apoptosis than A20/vector cells over the 3- to 6-week time course of tumor development.

In summary, we have shown that Rap activation has a key role in the dissemination of B-cell lymphomas *in vivo*, suggesting that Rap and its downstream effectors may be good therapeutic targets for reducing the spread of B-cell lymphomas to critical organs.

Conflict of interest

The authors declare no conflict of interest.

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