The adenomatous polyposis coli-associated exchange factors Asef and Asef2 are required for adenoma formation in *Apc*^{Min/+} mice

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Sporadic and familial colorectal tumours usually harbour biallelic adenomatous polyposis coli (APC)-associated mutations that result in constitutive activation of Wnt signalling. Furthermore, APC activates Asef and Asef2, which are guanine-nucleotide exchange factors specific for Rac1 and Cdc42. Here, we show that Asef and Asef2 expression is aberrantly enhanced in intestinal adenomas and tumours. We also show that deficiency of either Asef or Asef2 significantly reduces the number and size of adenomas in *Apc*^{Min/+} mice, which are heterozygous for an APC mutation and spontaneously develop adenomas in the intestine. We observed that the APC–Asef/Asef2 complex induces c-Jun amino-terminal kinase-mediated transactivation of matrix metalloproteinase 9, and is required for the invasive activity of colorectal tumour cells. Furthermore, we show that Asef and Asef2 are required for tumour angiogenesis. These results suggest that Asef and Asef2 have a crucial role in intestinal adenoma formation and tumour progression, and might be promising molecular targets for the treatment of colorectal tumours.

Keywords: APC; Asef; invasion; metalloproteinase; tumour

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INTRODUCTION

Inactivation of the tumour suppressor adenomatous polyposis coli (APC) by biallelic mutations is responsible for sporadic and familial colorectal tumours (Fodde *et al*, 2001). APC induces the degradation of β -catenin and negatively regulates Wnt signalling; APC recruits β -catenin into the multi-protein complex that

contains axin—or the closely related axin 2/conductin/axil—glycogen synthase kinase 3 β and casein kinase 1 α , and induces its proteasome-mediated degradation. The truncated mutant APCs identified in colon tumours are defective in this activity and, as a result, β -catenin levels are elevated and Wnt signalling is constitutively activated in colorectal tumour cells. It has been shown that the interaction of phosphorylated c-Jun with transcription factor 4 is required for this activation of Wnt signalling and intestinal cancer development in *Apc*^{Min/+} mice (Nateri *et al*, 2005). Although it is widely accepted that the ability of APC to negatively regulate Wnt signalling is essential for its tumour suppressor function, most mutations in colorectal tumours occur in APC and only a small percentage of mutations occur in β -catenin (Fodde *et al*, 2001). It is therefore possible that inactivation of the additional APC functions might also be important for colorectal tumorigenesis. In this regard, it is interesting that APC has recently been shown to interact with cellular proteins other than β -catenin, including Asef and Asef2, and to regulate cytoskeletal networks (Akiyama & Kawasaki, 2006).

Asef and Asef2 are guanine-nucleotide exchange factors specific for Rac1 and Cdc42 (Kawasaki *et al*, 2000, 2003, 2007; Gotthardt & Ahmadian, 2007; Hamann *et al*, 2007). APC enhances the guanine-nucleotide exchange factor activity of Asef and Asef2 by binding to their amino-terminal regions and regulates cell morphology, adhesion and migration. Furthermore, we have recently generated Asef-deficient mice and observed that Asef is important for tumour angiogenesis (Y.K. & T.A., unpublished data). These findings raise the possibility that Asef and Asef2 might be important for adenoma formation as well as tumour progression to invasive malignancy.

In this study, we generated Asef2-deficient mice and crossed Asef-deficient and Asef2-deficient mice with *Apc*^{Min/+} mice. We show here that Asef and Asef2 are required for adenoma formation in *Apc*^{Min/+} mice. Furthermore, we show that Asef and Asef2 are involved in tumour invasion. Our results suggest that compounds targeting Asef and Asef2 might hold promise as new anti-tumour reagents.

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Fig 1 | Analysis of *Asef* and *Asef2* expression levels in colon cancer tissues. (A) Quantitative analysis of *Asef*, *Asef2*, *MMP9* and *axin 2* expression in human colon cancerous and corresponding non-cancerous tissues by real-time RT-PCR. *Asef*, *Asef2*, *MMP9* and *axin 2* mRNA expression was quantified as the percentage relative to *GAPDH* mRNA ($n = 19$ pairs). Filled circles represent cancerous tissues in which the indicated mRNA is expressed at a higher level than in the corresponding non-cancerous tissues. For *Asef*, the open circles in the bottom row (Neg) represent tissues in which *Asef* expression was not detectable. *Axin 2* was used as a marker of β -catenin-TCF-mediated activation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (B) Immunohistochemical analysis of *Asef/Asef2* on sections from human colon cancer tissues and adjacent normal mucosa. The antibody used here recognizes both *Asef* and *Asef2*. Scale bar, 50 μ m. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA; RT-PCR, reverse transcriptase-PCR; TCF, T-cell factor.

RESULTS AND DISCUSSION

Asefs expression in human colorectal tumours

We examined ASEF and ASEF2 expression in human colorectal tumours and adjacent non-cancerous tissues by using real-time reverse transcriptase-PCR (RT-PCR) and immunohistochemical analyses. ASEF was highly expressed in most of the colorectal tumours examined, whereas it was expressed at low levels in most of the corresponding non-cancerous tissues (Fig 1A,B). The expression of ASEF2 was also significantly higher in colorectal tumours than in the non-cancerous tissues. Thus, ASEF and ASEF2 expression is aberrantly enhanced in most human colorectal tumours.

Roles of Asefs in intestinal adenoma formation

To investigate the significance of *Asef* and *Asef2* in intestinal tumorigenesis, we generated *Asef2*^{-/-} and *Asef*^{-/-}*Asef2*^{-/-} mice (supplementary Fig S1A–E online). All of the mice, including *Asef*^{-/-} mice, seemed to be morphologically normal, had a lifespan comparable with that of wild-type mice and were fertile. A histological analysis of the intestine and other organs, including the lung, liver and spleen, revealed no structural differences among wild-type, *Asef*^{-/-}, *Asef2*^{-/-} and *Asef*^{-/-}*Asef2*^{-/-} mice (supplementary Fig S2A,B online; S.T., Y.K. & T.A., unpublished data). Furthermore, analyses of intestinal differentiation markers, including lysozyme staining of Paneth cell granules, periodic acid Schiff staining of mucin production in goblet cells and alkaline phosphatase staining of enterocytes revealed no differences (supplementary Fig 2A online and unpublished data). In addition, immunostaining using Ki67 and caspase-3 antibodies revealed no difference in cell proliferation or survival (supplementary Fig S2A online; S.T., Y.K. & T.A., unpublished data). Furthermore, a bromodeoxyuridine-chasing experiment revealed no difference in cell migration (supplementary Fig S3 online).

Thus, *Asef* and *Asef2* deficiencies do not seem to affect intestinal structure, differentiation or physiology.

When we crossed *Asef*^{-/-} mice with *Apc*^{Min/+} mice, we saw that homozygous *Asef* deficiency significantly reduced the number and size of adenomas in *Apc*^{Min/+} mice (Fig 2A–C). We also crossed *Asef2*^{-/-} mice with *Apc*^{Min/+} mice and observed that homozygous *Asef2* deficiency had an even more pronounced effect on adenoma formation in *Apc*^{Min/+} mice than the *Asef* deficiency. Furthermore, *Asef2*^{-/-}*Apc*^{Min/+} mice survived significantly longer than *Apc*^{Min/+} mice (Fig 2D). *Apc*^{Min/+} mice heterozygous for either *Asef* or *Asef2* exhibited an intermediated phenotype (Fig 2C,D), suggesting that the differences in number and size of adenoma depend on the levels of *Asef* or *Asef2* expression. Furthermore, we observed that *Apc*^{Min/+} mice deficient in both *Asef* and *Asef2* developed only a few small adenomas (Fig 2A–C). The effects of *Asef* and *Asef2* deficiencies seem to be additive. The wild-type *Apc* was lost in these small adenomas (Y.K. & T.A., unpublished data). These results suggest that *Asef* and *Asef2* have crucial functions in intestinal adenoma formation. In addition, p21-activated kinase (PAK) assays revealed that the amounts of active guanosine triphosphate-bound Rac1 and Cdc42 were lower in adenomas of *Asef*^{-/-}*Asef2*^{-/-}*Apc*^{Min/+} mice than those of *Apc*^{Min/+} mice (Fig 2E). Thus, Rac1 and Cdc42 signalling might be reduced in adenomas of *Asef*^{-/-}*Asef2*^{-/-}*Apc*^{Min/+} mice compared with those of *Apc*^{Min/+} mice.

Asefs upregulate MMP9 expression via the JNK pathway

We attempted to examine the mechanisms by which *Asef* and *Asef2* contribute to intestinal adenoma formation in the context of the role of matrix metalloproteinase (MMP) expression and angiogenesis. As Rac1 and Cdc42 are known to activate the c-Jun N-terminal kinase (JNK) pathway, and thereby trans-activate c-Jun target genes, including *MMP9*, *MT1-MMP* and

COX2 (Davis, 2000; Johnson & Lapadat, 2002), we investigated whether MMP expression is enhanced by overexpression of Asef. RT-PCR analysis showed that the expression of *MMP9*, but not of *MMP7*, *MT1-MMP* or *COX2*, was increased when Madin–Darby canine kidney (MDCK) epithelial cells were transfected with *Asef-ΔAPC*, a mutant that lacks the N-terminal APC-binding region (ABR) and has stronger guanine-nucleotide exchange factor activity than wild-type Asef (supplementary Fig S4A,B online; K.M., Y.K. & T.A., unpublished data; Kawasaki *et al*, 2000). Immunoblotting analysis using an MMP9 antibody revealed that MMP9 protein expression is also increased in response to overexpression of *Asef-ΔAPC* (K.M., T.K. & T.A., unpublished data). By contrast, there was little induction of *MMP9* expression by *Asef-ΔDH*, a mutant lacking the DH domain. Wild-type Asef had a weaker effect than *Asef-ΔAPC*, but its activity was increased when co-transfected with *APC-1309*, a truncated mutant APC identified in colorectal tumours (supplementary Fig S4A,B online). However, co-expression of wild-type APC with Asef was less effective than *APC-1309*.

Consistent with these results, the amount of activated JNK1—that is, JNK1 phosphorylated at Thr 183 and Tyr 185 (pJNK1)—but not of pJNK2, was increased in MDCK cells transfected with *Asef-ΔAPC*, or with Asef and full-length APC or *APC-1309* (supplementary Fig S4C online). By contrast, the amounts of JNK1, p38, extracellular-signal-regulated kinase (ERK) and phosphorylated active ERK (pERK) were not increased, whereas the amount of phosphorylated active p38 (pp38) increased slightly. In addition, a reporter assay revealed that Asef transactivates the c-Jun promoter (K.M., Y.K. & T.A., unpublished data). When we performed similar experiments using Asef2, we obtained similar results (supplementary Fig S4A–C online). Furthermore, treatment of cells with a JNK inhibitor, SP600125, resulted in the inhibition of *Asef-ΔAPC*-induced upregulation of *MMP9*, whereas inhibitors of ERK-activating kinase and p38, PD98059 and SB203580, had undetectable and weak effects, respectively (supplementary Fig S4D online). Taken together, these results suggest that Asef and Asef2 have the potential to upregulate MMP9 expression mainly through the JNK signalling pathway.

Next, we examined the expression levels and gelatinolytic activities of MMP9 in adenomas generated in *Apc^{Min/+}*, Asef-deficient, and Asef- and Asef2-deficient *Apc^{Min/+}* mice. Immunoblotting, immunohistochemistry and zymogram analysis revealed that the expression levels of pJNK1 and phosphorylated active c-Jun, but not of JNK1 and c-Jun, and the gelatinolytic activity of MMP9 were increased in adenomas compared with the adjacent normal tissues of *Apc^{Min/+}* mice (Fig 2F,G). These increases were more prominent in larger adenomas. However, these alterations were barely observed in adenomas of either Asef-deficient or Asef- and Asef2-deficient *Apc^{Min/+}* mice. However, the levels of c-Myc, as well as pERK and pp38, were enhanced in adenomas of both *Apc^{Min/+}* and Asef-deficient *Apc^{Min/+}* mice compared with the normal tissues (Fig 2G). Furthermore, immunohistochemical analysis revealed nuclear accumulation of β-catenin in adenomas of both *Apc^{Min/+}* and *Asef^{-/-}Asef2^{-/-}Apc^{Min/+}* mice (supplementary Fig S5 online). Thus, Wnt signalling is activated in adenomas of Asef-deficient and Asef- and Asef2-deficient *Apc^{Min/+}* mice. These results suggest that Asef and Asef2 upregulate MMP9 expression through the JNK signalling pathway in adenomas of *Apc^{Min/+}* mice.

In addition, consistent with the results obtained from studies on human colorectal cancers, Asef and Asef2 expression was seen to be enhanced in adenomas of *Apc^{Min/+}* mice compared with the adjacent normal tissues (Fig 2F,G). We found that overexpression of an active mutant of β-catenin, β-catenin-S33Y, into HeLa and human embryonic kidney 293 cells did not result in any increase in Asef and Asef2 messenger RNA levels (supplementary Fig S6 online). Thus, the mechanism of this Asef and Asef2 upregulation does not involve Wnt signalling and remains to be investigated.

It is known that MMP9 is highly expressed in colorectal tumour cells (Coussens *et al*, 2002; Egeblad & Werb, 2002; Fig 1A). To test whether Asef, Asef2 and APC are involved in MMP9 expression in these cells, we knocked down Asef, Asef2 or APC in SW480 and SW620 (human colon cancer) cell lines that contain truncated mutant APCs, by using short hairpin RNAs (supplementary Fig S7 online). Knockdown of Asef, Asef2 or APC resulted in significant decreases in the level of MMP9 expression and gelatinolytic activity, as well as pJNK1 expression in SW480 and SW620 cells, but not in the HCT116 (colon carcinoma) cell line, which contain wild-type APC and mutated β-catenin (Fig 3A). Thus, Asef and Asef2 activated by truncated mutant APCs might be involved in enhanced MMP9 expression in SW480 and SW620 cells. Consistent with this idea, we saw that overexpression of *APC-1309* along with Asef in COS-7 and HeLa cells resulted in an increase in the amounts of the active guanosine triphosphate-bound forms of Rac1 and Cdc42, respectively (supplementary Fig S8 online). However, wild-type APC does not seem to have a crucial function in MMP9 expression in HCT116 cells. We observed that the amount of truncated mutant APCs, associated with Asef in SW480 and SW620 cells, is much higher than that of wild-type APC associated with Asef in HCT116 cells, reflecting the amount of truncated mutant APCs and wild-type APC expressed in these cells (supplementary Fig S9 online). In addition, treatment of SW480 cells with the JNK inhibitor SP600125 resulted in an inhibition of MMP9 expression, whereas the ERK-activating kinase inhibitor PD98059, and the p38 inhibitor SB203580, had undetectable and weak effects, respectively (Fig 3B). These results suggest that Asef, Asef2 and APC are required for MMP9 upregulation mainly through the JNK signalling pathway in colorectal tumour cells. We obtained similar results using Asef–ABR, a construct that acts as a dominant-negative mutant competing with Asef for binding to APC (supplementary Fig S10A online; Kawasaki *et al*, 2003). Thus, the interaction between Asef/Asef2 and truncated mutant APC might be crucial for MMP9 upregulation in colorectal tumour cells.

MMP9 and invasion of colorectal tumour cells

MMP9 is known to have crucial roles in both the development of benign lesions and in late-stage tumour progression, invasion and metastasis (Coussens *et al*, 2002; Egeblad & Werb, 2002). To address the significance of Asef-induced, Asef2-induced and APC-induced MMP9 expression in the invasive activity of colorectal tumour cells, we performed Matrigel invasion assays. Knockdown of either Asef, Asef2 or APC resulted in significant decreases in the invasive activities of SW480 and SW620 cells, but not of HCT116 cells (Fig 4A). Overexpression of MMP9 restored the invasive activity of SW480 cells in which Asef2 or APC had been knocked down (Fig 4B). We obtained similar results

using the dominant-negative mutant Asef-ABR (supplementary Fig S10B online). These results suggest that Asef-mediated, Asef2-mediated and APC-mediated upregulation of MMP9 is essential for the invasion of colorectal tumour cells.

To elucidate the relevance of MMP9 upregulation in adenoma formation, we assessed the effect of zoledronic acid (ZA), a specific inhibitor of MMP9 (Giraud *et al*, 2004). Treatment of *Apc^{Min/+}* mice with ZA for 6 weeks resulted in a significant reduction in the number

Fig 2 | Absence of Asef and/or Asef2 impairs intestinal adenoma formation in $Apc^{Min/+}$ mice. (A) Macroscopic comparison of the small intestine of $Apc^{Min/+}$ (left) and $Asef^{-/-}Asef2^{-/-}Apc^{Min/+}$ (right) mice at 4 months of age. Arrowheads and arrows indicate large and small adenomatous polyps, respectively. (B) Haematoxylin and eosin staining of intestinal 'Swiss roll' sections from $Apc^{Min/+}$ and $Asef^{-/-}Asef2^{-/-}Apc^{Min/+}$ mice at 4 months of age. The regions in boxes are magnified and shown in the lower panels. The dashed lines depict the boundary between normal and adenoma tissues. (C) The mean numbers and sizes of intestinal polyps in $Apc^{Min/+}$ mice with different Asef and Asef2 genotypes at 4 months of age ($n = 8-18$ per genotype). * $P < 0.05$; ** $P < 0.001$. (D) Kaplan-Meier survival curves of $Asef2^{+/+}Apc^{Min/+}$, $Asef2^{+/-}Apc^{Min/+}$ and $Asef2^{-/-}Apc^{Min/+}$ mice ($n = 24-27$ per genotype). (E) The amounts of GTP-bound Rac1 and Cdc42 in polyps of $Apc^{Min/+}$ and $Asef^{-/-}Asef2^{-/-}Apc^{Min/+}$ mice. GTP-bound GTPases were precipitated using GST-PAK CRIB coupled to glutathione-Sepharose beads and detected by immunoblotting with the indicated antibodies (for Rac1 and Cdc42). The total amount of GTPases in the lysates before pulldown assays were also analysed. (F) Immunohistochemical analysis of pJNK, MMP9 and Asef/Asef2 in intestinal sections from $Apc^{Min/+}$ and $Asef^{-/-}Asef2^{-/-}Apc^{Min/+}$ mice. Arrowheads indicate pJNK staining in intestinal crypts (also see supplementary Fig S12 online). Scale bar, 100 μ m. (G) Immunoblotting analysis and zymography of the normal intestinal mucosa (N) and polyps (P) of $Apc^{Min/+}$ and $Asef^{-/-}Apc^{Min/+}$ mice. Tissue lysates were subjected to immunoblotting with antibodies specific for the indicated proteins. Polyps of similar sizes of $Apc^{Min/+}$ and $Asef^{-/-}Apc^{Min/+}$ mice were used for experiments. As pJNK2 showed no change in abundance, only the bands of pJNK1 and JNK1 are shown in this figure. Actin and c-Myc were used as a loading control and a marker of β -catenin-TCF-mediated activation, respectively. MMP9 gelatinolytic activities were examined by gelatin zymography. CRIB, Cdc42/Rac interactive binding; GST, glutathione S-transferase; GTP, guanosine triphosphate; MMP9, matrix metalloproteinase 9; PAK, p21-activated kinase; pJNK, phosphorylated c-Jun N-terminal kinase; TCF, T-cell factor; zymo, gelatin zymography.

Fig 3 | Asef, Asef2 and APC are required for JNK phosphorylation and MMP9 expression in colorectal tumour cells. (A) Cells were transfected with shRNA expression vectors encoding the indicated shRNAs and subjected to immunoblotting with antibodies to pJNK1 or JNK1 (top two panels) and to semi-quantitative RT-PCR (middle two panels). Secretion of MMP9 was assayed by gelatin zymography (lower panel). SW480 and SW620 cells contain truncated APC, whereas HCT116 cells contain wild-type APC and mutated β -catenin. (B) The effects of the JNK inhibitor SP600125 on MMP9 secretion in SW480 cells. APC, adenomatous polyposis coli; MMP9, matrix metalloproteinase 9; JNK, c-Jun N-terminal kinase; RT-PCR, reverse transcriptase-PCR; shRNA, short hairpin RNA; zymo, gelatin zymography.

of adenomas (Fig 5A). By contrast, when $Asef^{-/-}Asef2^{-/-}Apc^{Min/+}$ mice were treated with ZA, no further suppression of adenoma formation was observed (data not shown). Zymography of spleen lysates from mice that had been treated with ZA revealed that the

activity of MMP9 is inhibited by ZA treatment *in vivo* (Fig 5B). These results are consistent with the idea that Asef-mediated, Asef2-mediated and APC-mediated MMP9 upregulation might be important for adenoma formation in $Apc^{Min/+}$ mice.

Fig 4 | Asef-mediated, Asef2-mediated and APC-mediated upregulation of MMP9 is essential for invasion of colorectal tumour cells. (A) Cells were transfected with vectors expressing the indicated shRNAs and tested in invasion assays. Results obtained are expressed as the means $\pm$ s.e.m. of at least four independent experiments. ** $P < 0.01$; *** $P < 0.001$. (B) Exogenous expression of MMP9 restores the invasive activity of SW480 cells in which Asef2 and APC had been knocked down. SW480 cells were transfected with siRNAs for Asef2 or APC together with MMP9, and subjected to immunoblotting with the indicated antibodies, gelatin zymography for MMP9 and invasion assays. Transfection efficiency was routinely more than 70%. Results are expressed as the means $\pm$ s.e.m. of four independent experiments. * $P < 0.05$. APC, adenomatous polyposis coli; MMP9, matrix metalloproteinase 9; shRNA, short hairpin RNA; siRNA, small interfering RNA; zymo, gelatin zymography.

Crucial functions of Asefs in tumour angiogenesis

Angiogenesis is a crucial process in the development of adenomas in *Apc*^{Min/+} mice, as well as in various human tumours (Ferrara & Kerbel, 2005). We recently observed that Asef is required for basic fibroblast growth factor-induced and vascular endothelial growth factor-induced microvessel formation, and that retinal angiogenesis is impaired in *Asef*^{-/-} mice (Y.K., S.F. & T.A., unpublished data). Furthermore, we observed that the growth, as well as vascularity, of subcutaneously implanted tumours are markedly impaired in *Asef*^{-/-} mice compared with wild-type mice. Thus, Asef and Asef2 might have important functions in tumour angiogenesis. To prove this possibility, we examined the density of microvessels in adenomas from Asef- and Asef2-deficient *Apc*^{Min/+} mice. Immunohistochemical analysis using a CD31 antibody revealed that the density of microvessels in adenomas from Asef- and Asef2-deficient *Apc*^{Min/+} mice was markedly lower than that in adenomas from *Apc*^{Min/+} mice (supplementary Fig S11 online). Although tumour-associated macrophages have been shown to facilitate angiogenesis (Lewis & Pollard, 2006), we

saw that macrophages were recruited to adenomas and released vascular endothelial growth factor normally in *Asef*^{-/-}*Asef2*^{-/-}*Apc*^{Min/+} mice (data not shown). These results suggest that Asef and Asef2 might have crucial roles in tumour angiogenesis and that the growth of adenomas might be retarded, at least in part, due to the impairment of angiogenesis caused by Asef and Asef2 deficiency.

Asefs: promising targets for cancer therapeutics

Our results suggest that Asef and Asef2 have important functions in adenoma formation, as well as in the progression, invasion and metastasis of colorectal tumours. In adenomas of both *Apc*^{Min/+} and *Asef*^{-/-}*Asef2*^{-/-}*Apc*^{Min/+} mice, the wild-type allele of *Apc* is lost, β -catenin is accumulated in the nucleus and Wnt target genes such as *c-Myc* are transactivated. Thus, Asef and Asef2 might synergize with aberrant Wnt signalling in adenoma formation.

We observed that Asef and Asef2 induce the expression of MMP9, which is known to induce extracellular matrix breakdown

Fig 5 | Involvement of MMP9 in intestinal adenoma formation. (A) The effects of the MMP9-specific inhibitor, ZA, on intestinal adenoma formation in $Apc^{Min/+}$ mice ($n = 5-7$ per genotype). * $P < 0.05$. (B) Suppression of MMP9 gelatinase activity by ZA *in vivo*. Lysates from spleens of control and ZA-treated $Apc^{Min/+}$ mice were subjected to gelatin zymography for MMP9. MMP9, matrix metalloproteinase 9; ZA, zoledronic acid; zymo, gelatin zymography.

and remodelling, and promote tumour cell motility and tumour angiogenesis (Coussens *et al*, 2002; Egeblad & Werb, 2002). MMPs are known to be crucial not only for late-stage tumour progression, invasion and metastasis, but also for the development of benign lesions (Wilson *et al*, 2003). Thus, Asef-mediated and Asef2-mediated induction of MMP9 might be crucial not only for tumour progression, invasion and metastasis, but also for adenoma formation. Consistent with our results, it has recently been reported that deficiency of MMP9, but not of MMP2, MMP12 or MMP19, reduces the number of adenoma in $Apc^{Min/+}$ mice (Sinnamon *et al*, 2008). Moreover, we found that Asef-induced and Asef2-induced MMP9 expression is mediated through the JNK-phosphorylated c-Jun pathway. This finding seems to be consistent with the fact that c-Jun is required for adenoma formation in $Apc^{Min/+}$ mice (Nateri *et al*, 2005). Interestingly, Wnt signalling is known to induce the expression of MMP7, but not of MMP9. Thus, it seems that Asef/Asef2 and Wnt signalling complement each other. Furthermore, we found that Asef and Asef2 have crucial functions in angiogenesis during adenoma formation. It is possible that MMP9 is involved in Asef-mediated and Asef2-mediated angiogenesis. Our results are consistent with the fact that angiogenesis is crucial not only for the later stages of tumour progression, but also for adenoma formation (Ferrara & Kerbel, 2005). Thus, these activities of Asef and Asef2 might contribute to adenoma formation, as well as to tumour progression. We envision that Asef and Asef2 could be promising molecular targets for therapy of colorectal tumours. As Asef-deficient and Asef2-deficient mice appear normal and have a lifespan comparable with that of wild-type mice, compounds targeting Asef and Asef2 would be expected to have few serious side effects.

METHODS

In vitro invasion assays. These were performed using 24-well Biocoat Matrigel invasion chambers (BD Biosciences, San Jose, CA, USA). Colorectal tumour cells (2.5×10^5 cells per well) transfected with plasmids were allowed to migrate to the underside of the top chamber for 24 h. SW480 cells were allowed to invade for 32 h. The lower chamber was filled with normal

medium containing 10 μ g/ml collagen type I (Koken, Tokyo, Japan) and 0.1% bovine serum albumin.

Mice. C57BL/6J- $Apc^{Min/+}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The Asef and Asef2 knockout lines were backcrossed for six generations onto C57BL/6J before mating. $Asef^{-/-}Apc^{Min/+}$, $Asef2^{-/-}Apc^{Min/+}$ and $Asef^{-/-}Asef2^{-/-}Apc^{Min/+}$ mice were generated by interbreeding Asef-deficient and/or Asef2-deficient mice and $Apc^{Min/+}$ mice.

Enumeration of intestinal polyps. Complete intestines, from stomach to rectum, were extracted, washed in phosphate-buffered saline and stained with methylene blue, and the number and diameter of polyps were scored under a dissection microscope.

Histology, immunohistochemistry and statistical analysis. Paraffin-embedded sections were stained with haematoxylin and eosin stain and with antibodies indicated in figures using the ABC Staining Kit (Vector Laboratories, Burlingame, CA, USA). Frozen sections were subjected to immunofluorescent staining with antibodies to CD31. We used the Periodic acid Schiff staining system (Sigma, St Louis, MO, USA). Statistical analysis was performed using the Mann-Whitney *U*-test and Student's *t*-test. A *P*-value < 0.05 was considered to be statistically significant.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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