Raf-1 sets the threshold of Fas sensitivity by modulating Rok-α signaling

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Abstract: Ablation of the Raf-1 protein causes fetal liver apoptosis, embryonic lethality, and selective hypersensitivity to Fas-induced cell death. Furthermore, Raf-1-deficient cells show defective migration as a result of the deregulation of the Rho effector kinase Rok-α. In this study, we show that the kinase-independent modulation of Rok-α signaling is also the basis of the antiapoptotic function of Raf-1. Fas activation stimulates the formation of Raf-1–Rok-α complexes, and Rok-α signaling is up-regulated in Raf-1-deficient cells. This leads to increased clustering and membrane expression of Fas, which is rescued both by kinase-dead Raf-1 and by interfering with Rok-α or its substrate ezrin. Increased Fas clustering and membrane expression are also evident in the livers of Raf-1-deficient embryos, and genetically reducing Fas expression counteracts fetal liver apoptosis, embryonic lethality, and the apoptotic defects of embryonic fibroblasts. Thus, Raf-1 has an essential function in regulating Fas expression and setting the threshold of Fas sensitivity during embryonic life.

Introduction

The Raf-1 kinase has been regarded for decades as one of the cornerstones of the Ras–Raf–MEK–ERK cascade, a highly conserved multipurpose signaling module implicated in cell proliferation, activation, survival, and oncogenesis. Genetic ablation of c-raf-1, however, has revealed that the essential function of this protein during embryonic life is not to activate the mitogen and extracellular regulated kinase (ERK) kinase (MEK)–ERK module and promote proliferation, but rather to prevent apoptosis (Huser et al., 2001; Mikula et al., 2001). Raf-1-deficient embryos die in utero between embryonic day (E) 10 and 12.5 with placental defects and increased apoptosis. In cultured embryonic fibroblasts, this survival function is selective in that Raf-1 protects the cells against Fas-induced apoptosis that is induced by Fas activation but not by TNFα. This is surprising, as the two receptors share most of their downstream effector mechanisms. A number of Raf-1 downstream effectors with a potential impact on apoptosis have been described previously, including the MEK–ERK module and the antiapoptotic transcription factor NF-κB but also the proapoptotic BH3-only protein Bad (Baccarini, 2002). Raf-1 also interacts directly with c-FLIP (FADD-like ice–like inhibitory protein; Kataoka et al., 2000), a homologue of caspase-8 that is devoid of catalytic activity. The function of c-FLIP is controversial, as it has been described both as an inhibitor and an activator of caspase-8 downstream of death receptors, depending on the stoichiometry of the complex (Chang et al., 2002; Micheau et al., 2002). Finally, Raf-1 forms a complex with and inhibits the proapoptotic kinases ASK-1 (Chen et al., 2001; Micheau et al., 2002) and MST-2 (O’Neill et al., 2004). In both cases, the interaction results in the inhibition of the proapoptotic kinase, which occurs independently of Raf-1 kinase activity. MST-2 inhibition is selectively relevant in the context of Fas-induced apoptosis (O’Neill et al., 2004).

Fas is the best-characterized member of the TNF receptor (TNFR) superfamily. Like other members of this family, it is expressed as a preassociated trimer that is formed via an extracellular preligand-binding domain (Siegel et al., 2000). Ligand binding stimulates the formation of microaggregates followed by the actin-dependent recruitment of the downstream effectors to a receptor-associated death-inducing signaling complex (DISC). The key components of the DISC are the adaptor protein FADD (Fas-associating death domain–containing protein) and the zymogen of caspase-8, which is the main initiator caspase in Fas signaling (Peter and Krammer, 2003). In addition, the long form of c-FLIP (c-FLIPL) is recruited to the
Results

in embryonic development. Sensitivity in fibroblasts in vitro and in the fetal liver in vivo and Raf-1, by regulating the cytoskeleton, sets the threshold of Fas death in Raf-1–deficient mice. These results demonstrate that increased membrane expression of Fas is the cause of embryonic defects shown by embryonic fibroblasts. Thus, in the context of Raf-1 knockout (KO) cells. The goals of this study were to investigate the molecular basis of Fas hypersensitivity in Raf-1 KO cells and to determine the role of this defect in embryonic development.

We have recently shown that Raf-1 plays an essential role in maintaining the organization of the cytoskeleton and, thus, in cell polarity and migration (Ehrenreiter et al., 2005). This function of Raf-1 is kinase independent and consists in restraining Rho downstream signaling. Therefore, the link between the association of Fas with the actin cytoskeleton and the intensity of the apoptotic signal was particularly intriguing in the context of Raf-1 knockout (KO) cells. The goals of this study were to investigate the molecular basis of Fas hypersensitivity in Raf-1 KO cells and to determine the role of this defect in embryonic development.

We show that the molecular basis of Fas hypersensitivity is the lack of Fas internalization as a result of a defect in the regulation of ezrin that results in the accumulation of Fas on the membrane. Increased membrane expression of Fas can be observed in fibroblasts in vitro and in fetal liver cells in situ, and reducing Fas levels rescues fetal liver apoptosis, embryonic lethality, and the apoptotic defects shown by embryonic fibroblasts. Thus, increased membrane expression of Fas is the cause of embryonic death in Raf-1–deficient mice. These results demonstrate that Raf-1, by regulating the cytoskeleton, sets the threshold of Fas sensitivity in fibroblasts in vitro and in the fetal liver in vivo and that this function is essential during embryonic development.

Results

Raf-1–deficient mouse embryonic fibroblasts (MEFs) are selectively hypersensitive to apoptosis induced by Fas activation and showed altered Fas surface expression. 129/SvHsd:Bl6 Raf-1 KO MEFs are more sensitive than wild-type (WT) cells to apoptosis induced by an agonistic Fas antibody or by Fasl, but not by TNFα. MEFs were treated either with αFas, with recombinant FLAG-tagged FasL cross-linked with 1 μg/ml α-FLAG M2 antibody, or with recombinant mouse TNFα at the concentrations indicated for 22 h in the presence of 5 μg/ml Chx and 0.5% FCS. Cell death was determined by CytoTox 96 assay. The values represent the mean ± SD [error bars] of three independent cell lines. *, P < 0.02; **, P < 0.01, according to a t-test comparing KO with WT cells. (B) The surface expression of Fas but not of TNFRI is altered in Raf-1 KO MEFs. WT and KO cells were stained with FITC-conjugated αFas (left) or with hamster α-mouse TNFRI antibody followed by FITC-conjugated goat α-hamster antibody (right) and were analyzed by flow cytometry. Dashed lines, isotype control (iso). (C) The expression of Fas mRNA levels were determined by RT-PCR. The HPRT gene was used as a normalization control. –, negative control; M, DNA marker. Molecular mass markers (in kilodaltons, C; or bp, D) are shown on the left.

The surface expression of Fas and TNFRI, which was determined by FACS analysis, reflected the selective hypersensitivity of Raf-1–deficient MEFs to Fas activation. KO cells showed a 4.5–5-fold increase in Fas surface expression with respect to WT (Fig. 1 B). In contrast, the increase in the total amount of Fas in KO cells was ~1.5-fold as determined by immunoblotting or RT-PCR (Fig. 1, C and D). The expression of TNFRI was low and indistinguishable in cells of either genotype (Fig. 1 B).

Raf-1 KO MEFs of the 129/SvHsd background were hypersensitive to Fas stimulation and expressed more Fas than their WT counterparts, exactly like Raf-1–deficient MEFs of the 129/SvHsd:Bl6 background, excluding a possible influence of the background on these phenotypes (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200504137/DC1).
In the KO, however, ezrin was not recruited to microvilli, which is in line with previous data (Takeuchi et al., 2005). Upon Fas stimulation, WT cells produced long protrusions that were brightly stained with an antibody against phosphorylated ezrin (pT567), which was hardly detectable in unstimulated WT cells (Fig. 2 E). These structures are reminiscent of the uropods observed in T lymphocytes—long (at least one third of the whole cell body) and large bulbs transiently protruding from the cell surface—whose formation depends on the phosphorylation of ezrin on T567 (Lee et al., 2004). In T lymphocytes, functionally active Fas is recruited to the KO than to the WT Fas DISC. Expression of the DISC components was comparable in WT and KO cells (Fig. 3 A, right).

The interaction of Fas with the actin cytoskeleton, which is mediated by ezrin (Lozupone et al., 2004), is required for DISC formation and internalization (Algeciras-Schimnich et al., 2002). DISC formation in KO and WT MEFs was analyzed by exclusively isolating the Fas that bound to excess agonist in vivo. Consistent with the higher surface expression (Figs. 1 B and 2 A), much more agonist-bound Fas was recovered from KO than from WT cells (Fig. 3 A). The amount of DISC components (FADD, procaspase-8, and c-FLIP<sub>L</sub>) recovered from KO cells was higher than that recovered from WT MEFs. How- ever, considering the amount of agonist-bound receptor iso- lated, DISC formation on a per-receptor basis was inefficient in KO cells was more rapid and effective in Raf-1 KO fibroblasts, however, the amount of Fas was too low to be detectable. As previously described, in unstimulated KO fibroblasts, the actin was detected in a rim around the cells and occasionally in patches and clusters that became more prominent upon Fas stimulation (Fig. 2 A). In addition, Fas internalization was significantly reduced in KO fibroblasts (Fig. 2 B). These phenotypes suggested a possible defect in Fas-stimulated cytoskeletal rearrangement, particularly in view of the cytoskeletal anomalies reported in migrating Raf-1 KO cells (Ehrenreiter et al., 2005). Upon Fas stimulation, WT cells produced long protrusions that were brightly stained with an antibody against phosphorylated ezrin (pT567), which was hardly detectable in unstimulated WT cells (Fig. 2 E). These structures are reminiscent of the uropods observed in T lymphocytes—long (at least one third of the whole cell body) and large bulbs transiently protruding from the cell surface—whose formation depends on the phosphorylation of ezrin on T567 (Lee et al., 2004). In T lymphocytes, functionally active Fas colocalizes with ezrin in the uropodes (Parlato et al., 2000): in adherent Raf-1 WT fibroblasts, however, the amount of Fas was too low to be detectable. As previously described, in unstimulated KO fibroblasts, the actin was detected in a rim around the cells, and the vimentin cytoskeleton was disorganized (Ehrenreiter et al., 2005). Upon Fas stimulation, bright patches of actin appeared, which partially colocalized with Fas. In addition, the vimentin cytoskeleton collapsed and was visualized as a dense perinuclear structure and at the tips of the short protrusions induced by Fas in these cells (Fig. 2, C and D). Although full-fledged uropods could not be observed in KO fibroblasts, these small, Fas-induced protrusions may be interpreted as an attempt to form such structures. In contrast to the WT, unstimulated KO fibroblasts contained significant amounts of ezrin<sup>pT567</sup> (Figs. 2 E, top; and 4 D, bottom) localized to microvilli, which is in line with previous data (Takeuchi et al., 1994). In the KO, however, ezrin<sup>pT567</sup> staining increased and concentrated in large spots, which partially colocalized with Fas (Fig. 2 E). The presence of hyperphosphorylated ezrin in KO cells could be confirmed by immunoblotting (Fig. 3 B).

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The aforementioned data indicate that DISC formation is less efficient in Raf-1 KO cells. Regardless of this, however, the analysis of whole cell lysates revealed that the overall Fas- induced caspase activation, as detected by the cleavage of caspase-8, 3, and c-FLIP<sub>L</sub>, was more rapid and effective in Raf-1 KO than in WT cells (Fig. 3 B). Taking into account the defect in Fas internalization and the recovery of DISC components...
Cells were either left untreated (0) or were treated with 2 μg/ml α-Fas plus 5 μg/ml Chx. At the indicated times, the DISC was collected using protein A-Sepharose beads. The presence of Fas, FADD, c-FLIP, caspase-8, and actin was determined by immunoblotting. Pro-c8, caspase-8 precursor; c8 p43, caspase-8 cleavage product; FlpL-p43/p41, c-FLIP, cleavage product; *, unspecific band. (B) Caspase-8, c-FLIP, and caspase-3 are rapidly cleaved, and ezrin is hyperphosphorylated in KO MEFs treated with α-Fas. MEFs were treated with α-Fas/Chx as described in A. Whole cell lysates collected at the indicated times were analyzed by immunoblotting. Molecular mass markers are shown in kilodaltons on the left.

Figure 3. Inefficient DISC formation but increased caspase activation in Raf-1 KO cells. (A) DISC formation is inefficient in Raf-1–deficient MEFs. Cells were either left untreated (0) or were treated with 2 μg/ml α-Fas plus 5 μg/ml Chx. At the indicated times, the DISC was collected using protein A-Sepharose beads. The presence of Fas, FADD, c-FLIP, caspase-8, and actin was determined by immunoblotting. Pro-c8, caspase-8 precursor; c8 p43, caspase-8 cleavage product; FlpL-p43/p41, c-FLIP, cleavage product; *, unspecific band. (B) Caspase-8, c-FLIP, and caspase-3 are rapidly cleaved, and ezrin is hyperphosphorylated in KO MEFs treated with α-Fas. MEFs were treated with α-Fas/Chx as described in A. Whole cell lysates collected at the indicated times were analyzed by immunoblotting. Molecular mass markers are shown in kilodaltons on the left.

even at late time points (120 min after stimulation), these data suggest that hypersensitivity to Fas-induced apoptosis results from less efficient but prolonged signaling by surface receptors in Raf-1 KO cells.

Raf-1 associates with Rok-α upon Fas stimulation and modulates Rok-α downstream signaling, Fas surface expression, and sensitivity to Fas-induced apoptosis

We have previously shown that in unstimulated and migrating fibroblasts, the formation of a Raf-1–Rok-α complex restrains the activation of Rok-α independently of Raf-1 kinase activity (Ehrenreiter et al., 2005). Among the consequences of Rok-α hyperactivation in Raf-1 KO cells are the disorganization of the vimentin cytoskeleton, which results in the relocalization of Rok-α to the plasma membrane, and the constitutive phosphorylation of ezrin on T567 (Ehrenreiter et al., 2005). The rapid collapse of vimentin structures and the hyperphosphorylation of ezrin in Fas-stimulated KO cells, which are hallmarks of Rok-α activation, suggested that Raf-1 might serve a similar function during Fas stimulation. Indeed, Rok-α was quickly translocated to the membrane of KO cells upon Fas stimulation, where it often resided in structures similar to small blebs (Fig. 4 A, arrowheads). In contrast, in WT cells, Fas stimulation resulted in an even more defined localization of Rok-α to the vimentin cytoskeleton (Fig. 4 A). Consistent with a role of Raf-1 in the regulation of Rok-α activity and localization, increasing amounts of Rok-α were detectable in endogenous Raf-1 immunoprecipitates (IPs) from Fas-induced WT cells (Fig. 4 B). As described for migration, the stable expression of either kinase–competent (KC) or kinase–dead (KD) Raf-1 in KO cells rescued the increase in Fas expression (Fig. 4, C and D) and ezrin phosphorylation/localization (Fig. 4 D) as well as the cytoskeletal defects (Ehrenreiter et al., 2005). Consistently, both KC and KD clones showed normal sensitivity to Fas-mediated apoptosis. TNFα-induced apoptosis was not affected by the reintroduction of KC or KD Raf-1 (Fig. 4 E). Vector-transfected cells and KO MEFs behaved indistinguishably.

Rok-α–dependent ezrin deregulation is responsible for the defects in Fas clustering and internalization in Raf-1–deficient cells. To determine whether hyperactivation of Rok-α was responsible for the increased clustering of Fas and for Fas hypersensitivity in KO cells, we transfected Raf-1–deficient cells with plasmids expressing a dominant-negative (DN) form of this protein. KD Rok-α (eG–Rok-α KD) abrogated both ezrin phosphorylation and Fas clustering (Fig. 5, A and B); however, as a result of the toxicity of the construct, it was not possible to investigate its effects on Fas-induced apoptosis. Therefore, we used small interfering RNA (siRNA) to silence Rok-α in KO and WT fibroblasts. 72 h after transfection with Rok-α siRNA, the expression of Rok-α, but not of the related kinase Rok-β, was radically reduced in cells of either genotype (Fig. 5 C). Silencing Rok-α abrogated the hypersensitivity of KO cells to Fas-induced apoptosis (Fig. 5 D) and dramatically reduced Fas clustering and ezrin phosphorylation (Fig. 5 E). Scrambled (SCR) siRNA had no effect on Rok-α expression, Fas clustering, Fas-induced apoptosis, or ezrin phosphorylation (Fig. 5, C–E). To determine whether ezrin hyperphosphorylation was causally linked to increased Fas clustering and to hypersensitivity to Fas-induced apoptosis, we transfected WT and KO cells with DN ezrin (DN-Ez-eG or ezrin1–310), which interferes with the function of endogenous ERM and microvilli formation (Crepaldi et al., 1997). DN ezrin abrogated Fas clustering (Fig. 5 F) and significantly decreased Fas-induced apoptosis in KO cells while causing a reproducible, albeit not significant, increase in Fas-induced apoptosis in WT cells (Fig. 5 G, top). In addition, DN ezrin had opposite effects on the internalization in WT and KO cells: it decreased internalization in the former and increased it in the latter (Fig. 5 G, bottom). Together with the data in Figs. 2 E, 3 B, and 4 D, these results show that in WT cells, ezrin is a positive regulator of Fas internalization and that it may modulate apoptosis by regulating the amount of Fas available for further stimulation at the cell surface. In contrast, in KO fibroblasts, hyperphosphorylation of ezrin prevents internalization and prolongs the death signal, thereby determining the difference in Fas sensitivity between WT and KO cells.

Fas mutation rescues all apoptotic defects of Raf-1-deficient primary MEFs (PEFs)

If the amount of Fas they express is the basis of the hypersensitivity of KO cells to Fas-induced apoptosis, reducing Fas levels
should rescue the defects of Raf-1–deficient PEFs in culture. To assay this, we prepared PEFs from 129/SvHsd:Bl6 Raf-1 KO embryos that were heterozygous for the lpr mutation, which functionally inactivates Fas (Watanabe-Fukunaga et al., 1992). In Raf-1 KO PEFs, Fas accumulated at the membrane and was often organized in small patches and clusters (Fig. 6 A and Fig. S2 B), available at http://www.jcb.org/cgi/content/full/jcb.200504137/DC1), as observed in immortalized fibroblasts (Figs. 2 A and 4 D). lpr heterozygosity reduced the amount of total and membrane-associated Fas (Fig. 6, A and B). Raf-1 KO PEFs are hypersensitive to Fas–induced cell death (Fig. 6 F and Fig. S2 A) and fail to accumulate in culture as a result of increased spontaneous apoptosis (Mikula et al., 2001). These defects were rescued in c-raf-1−/−; lpr/+ cells (Fig. 6, D–F; KO; lpr/+), indicating that they are caused by alterations in Fas signaling. Because continuously growing KO and WT PEFs constitutively produce indistinguishable amounts of FasL (Fig. 6, B and C), the increase in spontaneous cell death shown by KO PEFs can be ascribed to Fas hypersensitivity (Fig. 6 E). Although KO; lpr/+ PEFs were not hypersensitive to Fas stimulation, Fas internalization was still perturbed in these cells (Fig. 6 G), and some Fas clustering could still be observed (Fig. 6 A). This confirms that Fas hypersensitivity is secondary to the cytoskeletal defects and that the net amount of Fas present on the cell surface is the crucial factor determining the differences between KO and WT cells.

**Fas or FasL mutations rescue midgestation lethality caused by c-raf-1 ablation**

The major defect of the Raf-1 KO embryos is increased fetal liver apoptosis. Therefore, we investigated whether Fas expression was altered in this organ. At E11.5–12.5, the fetal liver consists of developing hepatocytes and haematopoetic cells, both of which reportedly express Fas (Terada and Nakanuma, 1995; De Maria et al., 1999). Consistent with the results obtained in PEFs and MEFs, Fas was often visualized as a rim around the cells in Raf-1–deficient livers (Fig. 7 A). In addition, FACS analysis showed that KO fetal liver cells expressed higher amounts of surface Fas compared with WT cells (Fig. 7 B). The lpr allele decreased Fas staining in both WT (not depicted) and KO fetal liver (Fig. 7, A and B). Raf-1 ablation is embryonic lethal both on the 129/Ola:Bl6 and on the 129/SvHsd:Bl6 background (Huser et al., 2001; Mikula et al., 2001). Remarkably, lpr heterozygosity rescued the fetal liver apoptosis that is characteristic of 129/SvHsd:Bl6 KO embryos (Fig. 7 A). The lpr allele did not significantly alter the ratio of E11.5 c-raf-1−/− embryos/litter, which was already submendelian (15%; n = 289) in this background (Huser et al., 2001; Mikula et al., 2001). This indicates the presence of an earlier defect with limited penetrance, and it could be connected with the placental insufficiency caused by Raf-1 ablation (Huser et al., 2001; Mikula et al., 2001). All c-raf-1−/−: lpr/+ embryos, however, survived to term, whereas their Raf-1–deficient littersmates died within E12.5. Embryonic lethality could similarly be rescued by introducing a mutated fastl allele (Takahashi et al., 1994) in Raf-1 KO animals (unpublished data). Most likely as a consequence of the placental defects that persisted in the Raf-1 KO embryos heterozygous for lpr or gld (not depicted), the rescued Raf-1 KO pups were smaller than their control littersmates, but they were not anemic (Fig. 7 C). Their eyes were consistently open at birth, which is a phenotype associated with delayed epithelial cell migration (Zhang et al., 2003) and consistent with the cell-autonomous migration defect of Raf-1 KO cells (Ehrenreiter et al., 2005). The rescued Raf-1 KO
animals failed to thrive and died perinatally for reasons presently unknown. Thus, increased Fas surface expression correlates with liver apoptosis and embryonic lethality in Raf-1 KO embryos, and both can be relieved by reducing the amount of Fas. Like in fibroblasts, this does not rescue the defects associated with the cytoskeleton and migration, as the pups are born with their eyes open.

Discussion

Raf-1 has long been regarded as the downstream effector linking Ras activation to the MEK–ERK module in the context of proliferation, survival, and differentiation. Gene ablation experiments have shown that this function must be redundant and
ligand dependence is impressively illustrated in vivo by the tosis, but it enhances sensitivity upon Fas activation. This does not cause detectable caspase activation or set off apop-

genetic backgrounds in culture. The increase in Fas density per liver cells in situ as well as in PEFs and in MEFs of different 
tering of Fas, which can be observed in Raf-1–deficient fetal 
tosis correlates with increased membrane expression and clus-
its effects on the cytoskeleton, regulates Fas expression and, 
2005). In this study, we provide evidence that Raf-1, through 
eralization of the cytoskeleton in migrating cells (Ehrenreiter et al., 
have revealed that the essential role of Raf-1 is to restrain 
apoptosis in midgestation embryos in vivo as well as spontaneous 
and Fas-induced apoptosis in cultured MEFs. In addition, 
Raf-1 is required to modulate Rok-α signaling and the remod-
ing the cytoskeleton, regulates Fas expression and, 
thereby, sets the threshold of Fas sensitivity in fibroblasts. The 
hypersensitivity of Raf-1–deficient cells to Fas-induced apop-
thesis in WT MEFs results in decreased Fas internalization. The 
the opposite happens in KO cells (Fig. 3), in which ezrin is con-
stitutively phosphorylated and is hyperphosphorylated upon 
activation. These results indicate that a tight control of 
ezrin activation is necessary for correct Fas signaling. Together 
with the evidence in a previous study (Parlato et al., 2000), 
they suggest a model in which ezrin phosphorylation is neces-
sary for uropod development and Fas clustering and, in turn, 
for DISC formation. However, if too much ezrin is phosphor-
ylated or if ezrin phosphorylation persists for too long, large 
Fas clusters that cannot be internalized, ineffective DISC for-
mation, and inefficient substrate cleavage result (Fig. 8). Fas 
internalization requires both actin and caspase-8 activity (Algeciras-Schimnich et al., 2002), and it has been suggested that 
DISC internalization may depend on the degradation of sub-
strates involved in cortical actin reorganization by DISC-associa-
ted caspase-8. In this situation, an excess of ezrinT567, 
which acts as an actin filament/plasma membrane cross-linker 
and may, therefore, prevent cortical actin reorganization, is 
probably counterproductive (Bretsccher et al., 2002). Indeed, 
Fas-stimulated, caspase-dependent dephosphorylation of ezrin 
has been shown previously (Kondo et al., 1997). In WT fibro-
blasts, Fas activation increases the interaction between Raf-1 
and Rok-α and, thereby, prevents the hyperactivation of the 
latter kinase and its substrates, favoring cortical actin rearrangements 
and Fas internalization. Thus, Rok-α downstream signaling has an impact on both the strength and duration of 
the apoptotic signal, and restraining Rok-α is the common
molecular basis of the essential function of Raf-1 in apoptosis and migration.

The control of Fas expression and internalization via the modulation of Rok-α is one of three examples of how Raf-1 can counteract apoptosis by inhibiting another kinase. Strikingly, in all three cases, this function of Raf-1 is kinase independent. The two other kinases, ASK-1 (Chen et al., 2001; Yamaguchi et al., 2004) and MST-2 (O’Neill et al., 2004), can both be activated by Fas. Thus, Raf-1 ablation perturbs the Fas cascade at different levels: it lowers the threshold of Fas activation, thereby boosting Fas signaling in general, and it inhibits proapoptotic kinases, reducing their specific contribution to Fas-induced cell death (O’Neill et al., 2004). If we correct only the former defect by reducing Fas-FasL interaction in Raf-1–deficient cells (by interfering with Rok-α signaling or by lpr heterozygosity) and embryos (by lpr or gld heterozygosity), we rescue their hypersensitivity to Fas. This indicates that the defective control of Fas expression is the rate-limiting defect in Raf-1–deficient cells and embryos. Consistent with this scenario, mathematical modeling of Fas-induced apoptosis has recently identified the concentration of activated Fas as a critical factor in setting the threshold of Fas-induced apoptosis (Bentele et al., 2004).

Besides rescuing fetal liver apoptosis, a reduction in Fas–FasL expression also appears to restore normal fetal erythropoiesis, as lpr/+ or gld/+ (unpublished data) Raf-1–deficient embryos or pups are not anemic. We have previously demonstrated that the anemia is caused by accelerated erythroblast differentiation, secondary to premature caspase-associated differentiation (Kolbus et al., 2002). To date, the stimulus causing differentiation-associated caspase activation in erythroblasts is entirely unclear. It has, however, been shown that erythroid cells express Fas–FasL, whereby immature erythroblasts express the receptor and react to the ligand, and more mature cells express the ligand. The paracrine interaction between Fas and FasL is hypothesized to regulate erythroid homeostasis (De Maria et al., 1999). In line with this, increased numbers of erythroid progenitor cells have been observed in lpr/lpr or gld/gld mice (Schneider et al., 1999). Indeed, heterozygosity at the lpr or gld locus restores normal caspase activation and erythroid differentiation in Raf-1–deficient erythroblasts in vitro (unpublished data).

The finding that Raf-1 selectively regulates the sensitivity to Fas-mediated apoptosis has important biomedical implications. Fas-mediated apoptosis plays a capital role in liver physiology and disease. Fas up-regulation has been observed in alcohol-induced liver disease and viral hepatitis; in addition, cytotoxic T lymphocytes, which express FasL, may induce apoptosis in the course of viral hepatitis, autoimmune liver disease, and allograft rejection (Kanzler and Galle, 2000). In all of these conditions, Fas activation is undesirable, and knowledge of the processes regulating it might help to find ways of preventing it. On the other hand, apoptosis plays a very relevant role in the progression of hepatocellular carcinoma (Kanzler and Galle, 2000). During progression, these tumors down-regulate Fas expression and are therefore insensitive to FasL produced by T lymphocytes; in addition, the tumor cells express FasL, which enables them to kill the T lymphocytes. Thus, modulating Fas–FasL expression enables the tumors to bypass tumor surveillance. In light of these observations, signal transducers selectively modulating Fas-mediated apoptosis can be considered attractive therapeutic targets in a number of conditions. Mice harboring tissue-restricted or inducible c-raf-1 deletion will be instrumental in defining the role of Raf-1 in the regulation of Fas expression and signaling during tumor progression.

**Materials and methods**

**Mice**
craf-1−/− 129/SvHsd mice (Mikula et al., 2001) and lpr/lpr and gld/gld (C57Bl/6; gift of A. Martin-Villalaba, Deutschen Krebsforschungszentrum, Heidelberg, Germany) animals were housed in the animal facility of the Max F. Perutz Laboratories. PCR analysis of offspring was performed as previously described (Mixter et al., 1995; Van Parijs et al., 1998; Mikula et al., 2001).

**Cell isolation, culture, infection, and transfection**
PEFs were isolated as previously described (Mikula et al., 2001). PEFs and MEFs were cultured in DME supplemented with 10% FCS (GIBCO BRL). Stable cell clones expressing KC and KD Raf-1 have been previously described (Ehrenreiter et al., 2003). For transient transfections, cells were transfected with KD DN Rok-α (pxJ40-eGFP–Rok-α KD; gift of Thomas Leung, Institute of Molecular and Cell Biology, Singapore) and DN ezrin (DN-EzeG; gift of R. Lamb, Institute of Cancer Research, London, UK) or the corresponding empty vectors using LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. Apoptosis was induced by treatment with either Jo2 (BD Biosciences), recombiant FLAG-tagged FasL (gift of Jürg Tschopp, University of Lausanne, Lausanne, Switzerland) cross-linked with 1 μg/ml mFLAG M2 antibody (Sigma-Aldrich), or murine TNFα (Calbiochem), all in the presence of cycloheximide (Chx; Calbiochem). Chx prevents the expression of NF-κB target genes (Micheau et al., 2001) and was added to sensitize the relatively resistant MEFs to Fas- or TNFα-induced apoptosis. Cell death was measured using the CytoTox 96 NonRadiactive Cytotoxicity Assay (Promega).

**RNA isolation and RT-PCR**
RNA was prepared using the NucleoSpin Purification Kit (CLONTECH Laboratories, Inc.) according to the manufacturer’s instructions. cDNA was generated by reverse transcription using oligo(dT) as primer and M-MuLV Reverse Transcriptase (Fermentas). PCR reactions (94°C for 30 s, 55°C for 30 s, 72°C for 30 s).
Orescence analysis, 4-
ized as previously described (Ehrenreiter et al., 2005). For Fas immuno- 
(Mikula et al., 2001). Actin, vimentin cytoskeleton, and Rok-
Histological and TUNEL analysis were performed as described previously 
Histological, immunofluorescence, and TUNEL analysis 
were counted by two independent investigators. The pictures show repre-
sentative Z-stacks. 

Flow cytometric analysis 
Cells were stained with either FITC-conjugated Jo2 or αTNFRF antibody (hamster α-mouse; BD Biosciences) followed by a FITC-conjugated secondary 
antity (Jackson Immunoresearch Laboratories) in PBS, 0.5% BSA, 
and analyzed. 

Immunoprecipitation and immunoblotting 
Cell lysis and immunoblotting were performed as previously described 
(Mikula et al., 2001) using the following primary antibodies: αFas, α-actin, 
α-caspase-3, and α-caspase-8 (Santa Cruz Biotechnology, Inc); αFasL, 
α-Raf-1, α-Rok-α, α-Rok-β, and α-panERK (BD Biosciences); α-FADD 
(BioVision); α-FITC (Sigma-Aldrich); α-tubulin (Sigma-Aldrich); and α-ERK and 
α-ERK Cell Signaling). Raf-1 IPs were prepared in TBS TWEEN-20 lysis 
buffer (200 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Tri-
ton X-100, and protease inhibitors) as previously described (Ehrenreiter 
et al., 2005).

DISC isolation 
WT and KO MEFs were stimulated with 2 μg/ml αFas plus 5 μg/ml Chx 
at 37°C. At the indicated time points, cells were lysed in ice-cold TBS 
TWEEN-20 lysis buffer. Insoluble material was removed by centrifugation, 
and the DISC was collected using protein A-Sepharose beads. The IPs were 
was four times and eluted by boiling in SDS-PAGE sample buffer. 
Protein A-Sepharose beads incubated with lysates from unstimulated 
cells were used as a negative control.

Statistical analysis 
All values are expressed as means ± SD. P values were calculated using 
the unpaired two-tailed t test assuming unequal variances. A P value ≤ 
0.05 is considered statistically significant.

Online supplemental material 
Fig. S1 shows that 129/SvHsd Raf-1–deficient MEFs are selectively hyper-
sensitive to Fas stimulation and express increased amounts of surface Fas. 
Fig. S2 shows hypersensitivity to Fas-induced apoptosis, Fas clustering, 
and ezrin hyperphosphorylation in Raf-1–deficient 129/SvHsd PEFs.

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References

Algeciras-Schimnich, A., L. Shen, B.C. Barnhart, A.E. Murmann, J.K. 
Burkhardt, and M.E. Peter. 2002. Molecular ordering of the initial sig-
naling events of CD95. Mol. Cell. Biol. 22:207–220.

Baccarini, M. 2002. An old kinase on a new path: Raf and apoptosis. Cell Death 
Differ. 9:783–785.

Bentele, M., I. Lavrik, M. Ulrich, S. Stosser, D.W. Heermann, 
H. Kalthoff, P.H. Krammer, and R. Eils. 2004. Mathematical modeling 
thresholds mechanism in CD95-induced apoptosis. J. Cell Biol. 
166:839–851.

Breitoser, A., K. Edwards, and R.G. Fehon. 2002. ERM proteins and merlin: in-
tegrators at the cell cortex. Nat. Rev. Mol. Cell Biol. 3:586–599.

Chang, D.W., Z. Xing, Y. Pan, A. Algeciras-Schimnich, B.C. Barnhart, S. 
Yash-Ohda, M.E. Peter, and X. Yang. 2002. c-FLIP(L) is a dual 
function regulator for caspase-8 activation and CD95-mediated apoptosis. 
EMBO J. 21:3704–3714.

Chen, J., K. Fujii, L. Zhang, T. Roberts, and H. Fu. 2001. Raf-1 promotes 
cell survival by antagonizing apoptosis signal-regulating kinase 1 through 
MEK-ERK independent mechanism. Proc. Natl. Acad. Sci. USA. 98:7783–7788.

Crepaldi, T., A. Gauteiro, P.M. Comoglio, D. Louvard, and M. Arpin. 
1997. Ezrin is an effector of hepatocyte growth factor-mediated migration 
and morphogenesis in epithelial cells. J. Cell Biol. 138:423–434.

De Maria, R., U. Testa, L. Luchetti, A. Zeuner, G. Stassi, E. Pelosi, R. Riccioli, 
N. Felli, P. Samoggia, and C. Peschle. 1999. Apoptotic role of Fas/Fas 
ligand system in the regulation of erythropoiesis. Blood. 93:796–803.

Ehrenreiter, K., D. Piazzolla, V. Velamoor, I. Sobczak, J.V. Small, J. Takeda, T. 
Lenz, and M. Baccarini. 2005. Raf-1 regulates Rho signaling and cell 
migration. J. Cell Biol. 168:955–964.

Huser, M., J. Luckett, A. Chiocekes, K. Mercer, M. Iwobi, S. Giblett, X.M. 
Sun, J. Brown, R. Marais, and C. Pritchard. 2001. MEK kinase activity 
is not necessary for Raf-1 function. EMBO J. 20:1940–1951.

Kanzler, S., and P.R. Galle. 2000. Apoptosis and the liver. Semin. Cancer Biol. 
10:173–184.
Kataoka, T., R.C. Budd, N. Holler, M. Thome, F. Martinon, M. Imler, K. Burns, M. Hahne, N. Kennedy, M. Kovacsovics, and J. Tschopp. 2000. The caspase-8 inhibitor FLIP promotes activation of NF-κB and Erk signaling pathways. Curr. Biol. 10:640–648.

Kolbus, A., S. Pilat, Z. Husak, E.M. Deiner, G. Stengl, H. Beug, and M. Baccarini. 2002. Raf-1 antagonizes erythroid differentiation by restraining caspase activation. J. Exp. Med. 196:1347–1353.

Kondo, T., K. Takeuchi, Y. Doi, S. Yonemura, S. Nagata, and S. Tsukita. 1997. ERM (ezrin/radixin/moesin)-based molecular mechanism of microvillar breakdown at an early stage of apoptosis. J. Cell Biol. 139:749–758.

Lee, J.-H., T. Katakai, T. Hara, H. Gonda, M. Sugai, and A. Shimizu. 2004. Roles of p-ERM and Rho-ROCK signaling in lymphocyte polarity and uropod formation. J. Cell Biol. 167:327–337.

Lozupone, F., L. Lugini, P. Matarrese, F. Luciani, C. Federici, E. Issi, P. Margutti, G. Stassi, W. Malorni, and S. Fais. 2004. Identification and relevance of the CD95-binding domain in the N-terminal region of ezrin. J. Biol. Chem. 279:9199–9207.

Micheau, O., S. Lens, O. Gaide, K. Alevizopoulos, and J. Tschopp. 2001. NF-kappaB signals induce the expression of c-FLIP. Mol. Cell. Biol. 21:5299–5305.

Micheau, O., M. Thome, P. Schneider, N. Holler, J. Tschopp, D.W. Nicholson, C. Briand, and M.G. Grutter. 2002. The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. J. Biol. Chem. 277:45162–45171.

Mikula, M., M. Schreiber, Z. Husak, L. Kucerova, J. Ruth, R. Wieser, K. Zatloukal, H. Beug, E.F. Wagner, and M. Baccarini. 2001. Embryonic lethality and fetal liver apoptosis in mice lacking the c-raf-1 gene. EMBO J. 20:1952–1962.

Mixer, P.F., J.Q. Russell, F.H. Darie, and R.C. Budd. 1995. Decreased CD4+CD8− TCR-alpha beta cells in lpr/lpr mice lacking beta 2-microglobulin. J. Immunol. 154:2063–2074.

O’Neill, E., L. Rushworth, M. Baccarini, and W. Kolch. 2004. Role of the kinase MST2 in suppression of apoptosis by the proto-oncogene product Raf-1. Science. 306:2267–2270.

Parlato, S., A.M. Giannmarioli, M. Logozi, F. Lozupone, P. Matarrese, F. Luciani, M. Falchi, W. Malorni, and S. Fais. 2000. CD95 (APO-1/Fas) linkage to the actin cytoskeleton through ezrin in human T lymphocytes: a novel regulatory mechanism of the CD95 apoptotic pathway. EMBO J. 19:5123–5134.

Peter, M.E., and P.H. Krammer. 2003. The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ. 10:26–35.

Schneider, E., G. Moreau, A. Arnould, F. Vasseur, N. Khodabaccus, M. Dy, and S. Ezine. 1999. Increased fetal and extramedullary hematopoiesis in Fas-deficient C57BL/6-lpr/lpr mice. Blood. 94:2613–2621.

Siegel, R.M., J.K. Frederiksen, D.A. Zacharias, F.K. Chan, M. Johnson, D. Lynch, R.Y. Tsien, and M.J. Lenardo. 2000. Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. Science. 288:2354–2357.

Takahashi, T., M. Tanaka, C.I. Brannan, N.A. Jenkins, N.G. Copeland, T. Suda, and S. Nagata. 1994. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. Cell. 76:969–976.

Takeuchi, K., N. Sato, H. Kanahara, N. Funayama, A. Nagafuchi, S. Yonemura, and S. Tsukita. 1994. Perturbation of cell adhesion and microvilli formation by antisense oligonucleotides to ERM family members. J. Cell Biol. 125:1371–1384.

Terada, T., and Y. Nakanuma. 1995. Detection of apoptosis and expression of apoptosis-related proteins during human intrahepatic bile duct development. Am. J. Pathol. 146:671–74.

Van Parijs, L., D.A. Peterson, and A.K. Abbas. 1998. The Fas/Fas ligand pathway and Bcl-2 regulate T cell responses to model self and foreign antigens. Immunity. 8:265–274.

Watanabe-Fukunaga, R., C.I. Brannan, N.G. Copeland, N.A. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature. 356:314–317.

Yamaguchi, O., T. Watanabe, K. Nishida, K. Kashihase, Y. Higuchi, T. Takeda, S. Hikosho, S. Hirotsu, M. Asahi, M. Tanike, et al. 2004. Cardiac-specific disruption of the c-raf-1 gene induces cardiac dysfunction and apoptosis. J. Clin. Invest. 114:937–943.

Zhang, L., W. Wang, Y. Hayashi, J.V. Jester, D.E. Birk, M. Gao, C.-Y. Liu, W.W.-Y. Kao, M. Kare, and Y. Xia. 2003. A role for MEK kinase 1 in TGF-(beta)activin-induced epithelium movement and embryonic eyelid closure. EMBO J. 22:4443–4454.