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ORIGINAL ARTICLE

Axl/Gas6/NFκB signalling in schwannoma pathological proliferation, adhesion and survival

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TAM family receptor tyrosine kinases comprising Tyro3 (Sky), Axl, and Mer are overexpressed in some cancers, correlate with multidrug resistance and contribute to tumourigenesis by regulating invasion, angiogenesis, cell survival and tumour growth. Mutations in the gene coding for a tumour suppressor merlin cause development of multiple tumours of the nervous system such as schwannomas, meningiomas and ependymomas occurring spontaneously or as part of a hereditary disease neurofibromatosis type 2. The benign character of merlin-deficient tumours makes them less responsive to chemotherapy. We previously showed that, amongst other growth factor receptors, TAM family receptors (Tyro3, Axl and Mer) are significantly overexpressed in schwannoma tissues. As Axl is negatively regulated by merlin and positively regulated by E3 ubiquitin ligase CRL4DCAF1, previously shown to be a key regulator in schwannoma growth we hypothesized that Axl is a good target to study in merlin-deficient tumours. Moreover, Axl positively regulates the oncogene Yes-associated protein, which is known to be under merlin regulation in schwannoma and is involved in increased proliferation of merlin-deficient meningioma and mesothelioma. Here, we demonstrated strong overexpression and activation of Axl receptor as well as its ligand Gas6 in human schwannoma primary cells compared to normal Schwann cells. We show that Gas6 is mitogenic and increases schwannoma cell-matrix adhesion and survival acting via Axl in schwannoma cells. Stimulation of the Gas6/Axl signalling pathway recruits Src, focal adhesion kinase (FAK) and NFκB. We showed that NFκB mediates Gas6/Axl-mediated overexpression of survivin, cyclin D1 and FAK, leading to enhanced survival, cell-matrix adhesion and proliferation of schwannoma. We conclude that Axl/FAK/Src/NFκB pathway is relevant in merlin-deficient tumours and is a potential therapeutic target for schwannoma and other merlin-deficient tumours.

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Keywords: Gas6/Axl; NFκB; merlin; proliferation/adhesion/survival; schwannoma

INTRODUCTION

Tyro3 (Sky), Axl and Mer are members of the TAM family of receptor tyrosine kinases sharing vitamin K-dependent ligands Gas6 and Protein S. TAM family receptors can be activated in both ligand-dependent and ligand-independent manner, resulting in receptor dimerization and autophosphorylation.[1–4] TAM family receptors are overexpressed in cancers and are markers for poor prognosis and correlate with multidrug resistance.[5–7] They also contribute to tumourigenesis by regulating migration and invasion, angiogenesis, cell survival and tumour growth.[5,8,9]

Mutations in the gene coding for a tumour suppressor, merlin, cause the development of multiple tumours of the nervous system such as schwannomas, meningiomas and ependymomas occurring either spontaneously or as a hereditary disease, neurofibromatosis type 2. The benign character of merlin-deficient tumours makes them relatively unresponsive to conventional chemotherapy, leaving invasive surgery or radiosurgery as the main treatment options. However, these treatments carry significant risks, particularly when the tumours are close to important neurological structures or if the tumours are multiple as in neurofibromatosis type 2, and new therapeutic strategies are needed. Merlin is known to be involved in the regulation of a variety of cellular signalling pathways to regulate cell-matrix adhesion, proliferation and survival. As schwannoma is the most common tumour and hallmark for neurofibromatosis type 2, we have investigated the tumour pathobiology and merlin signalling pathways, using a human primary schwannoma *in vitro* model comprising human primary Schwann and schwannoma cells.[10–15]

We have previously shown that, in addition to the overexpression of multiple receptors, platelet-derived growth factor receptors,[10] insulin-like growth factor I receptor,[16] integrins[17] and ErbB2/3,[18] TAM family receptors are significantly overexpressed in schwannoma tissues.[11] The relevance of Axl in merlin-deficient tumours is underlined by findings showing that Axl is negatively regulated by merlin and positively regulated by E3 ubiquitin ligase CRL4DCAF1. Merlin seems to inhibit E3 ubiquitin ligase CRL4DCAF1, which is responsible for tyrosine kinase receptors expression changes in merlin-deficient tumours.[19] The ability of Axl to positively regulate oncogene Yes-associated protein, a downstream member of Hippo pathway known to be under merlin regulation in schwannoma and involved in increased proliferation of meningioma and mesothelioma, further support for a potential role of Axl in merlin-deficient tumours.[20] Moreover, Gas6 stimulates human Schwann cell proliferation *in vitro* via Axl and Tyro3.[21] We thus hypothesize that TAM family receptors may be important in merlin-deficient schwannoma pathology. We have therefore in this paper investigated the role of Gas6/Axl in schwannoma cell proliferation, cell-matrix adhesion and survival.

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Using our schwannoma *in vitro* model, we demonstrate strong overexpression of all three members of the TAM receptor family, and their ligand Gas6 in human schwannoma. We show that Gas6 is mitogenic and increases schwannoma cell-matrix adhesion and survival acting via Axl in schwannoma cells. Furthermore, Gas6 signalling via specifically Axl involves focal adhesion kinase (FAK) and Src, but not the ERK1/2, JNK1/2 and AKT signalling pathways. We also demonstrate the role of NFκB, which regulates Gas6/Axl-mediated overexpression of survivin, cyclin D1 and FAK leading to enhanced survival, cell-matrix adhesion and proliferation of schwannoma. NFκB expression was found to be merlin-dependent and its activity depended on Axl.

RESULTS

Axl, Tyro3 and Mer receptors are overexpressed in schwannoma

TAM family receptors, Axl, Tyro3 and Mer are expressed at higher levels in human primary schwannoma compared with normal Schwann cells (Figures 1a, c, and e). The expression of Axl and Tyro3 but not Mer was decreased by the reintroduction of merlin (Figures 1b, d and f). Thus Axl and Tyro3 but not Mer are under direct merlin control.

Gas6 is expressed and released from both schwannoma and Schwann cells

The TAM receptor ligand, Gas6, was also expressed at higher levels in schwannoma than in Schwann cells (Figure 1g). Furthermore, Gas6 was also released from both cell types, with slightly higher levels (not significant) being observed from schwannoma cells as compared with Schwann cells (Figure 1h). Moreover, Gas6 expression was decreased upon reintroduction of merlin, suggesting that this TAM ligand is regulated by merlin (Figure 1i).

Gas6 increases schwannoma proliferation, cell-matrix adhesion and survival and activates Axl receptor

Proliferation. Gas6-containing medium (40 ng/ml, 24 h) increased proliferation of schwannoma cells by ~80% with the same efficiency as 10% FCS, while mock had no effect (Figure 2a). The specificity of Gas6-mediated proliferation was confirmed using recombinant human Axl and Tyro3 ectodomain-Fc chimeras to capture Gas6 from media (Figure 2b), and the chimeras' specificities were confirmed by using Insulin-like growth factor-1 (IGF-I), to which they did not bind (Figure 2c). Axl shRNA completely inhibited the Gas6-mediated increase in proliferation of schwannoma cells (Figure 2d). Moreover, Gas6 increased the phosphorylation/activity of Axl (Figure 2e). Thus, Gas6-mediated proliferation of schwannoma cells is mediated via the Axl receptor.

Adhesion. Similarly to the increased proliferation, Gas6 (40 ng/ml) potentiated schwannoma cell-matrix adhesion by 40% (Figure 2f), while medium had no effect (Figure 2f). Axl shRNA completely inhibited the Gas6-mediated increased cell-matrix adhesion (Figure 2g). Thus, Gas6-mediated adhesion occurs via the Axl receptor. Controls for the specificity of Axl shRNA are shown in Figure 2h, where only Axl downregulation but not Tyro3 or Mer were observed (Figure 2h).

Survival. Schwannoma cells cultured, in DMEM without any supplements, for 5 days displayed only 6% of cell death, which was rescued by Gas6 40 ng/ml to ~2% ($n = 5$) (Figure 2i). The data suggest that schwannoma cells develop pro-survival mechanisms during starvation, which confirms our previous findings,[22] and that Gas6 seem to have a role in the increase of cell viability.

Tissue expression. As Gas6-mediated proliferation and cell-matrix adhesion are fully dependent on Axl receptor, we went on to explore Axl-mediated signalling in schwannoma. Firstly, by immunohistochemistry, we were able to show strong staining for both Axl (Figure 2j) and phospho-Axl (Figure 2k), in human schwannoma tissues compared with normal nerves, with both cytoplasmic and widespread nuclear immunoreactivity in the tumour cells. Next, we dissected potential downstream signalling pathways from Axl in human schwannoma primary cells.

Signalling pathways activated by Gas6 via Axl in schwannoma

Gas6 leads to the activation of ERK1/2, JNK, AKT, FAK and Src in schwannoma cells. Stimulation of schwannoma cells with Gas6 (40 ng/ml) for 5 min lead to strong activation/phosphorylation of ERK1/2, JNK and AKT (Figures 3a, b and c), which have previously been shown to contribute to increased proliferation, cell-matrix adhesion and survival in schwannoma.[10,11,23–27] FAK, which has been previously shown to be overexpressed and strongly activated in schwannoma, contributing to increased proliferation and cell-matrix adhesion,[10,12] is also activated/phosphorylated by Gas6 at autophosphorylation site Y397 and Src phosphorylation site Y925 (Figures 3d and e). Levels of active/phosphorylated Src P-Y416, previously demonstrated to have an important role in schwannoma development by increasing proliferation,[12] were also increased by Gas6 (Figure 3f).

Dissection of Gas6-mediated pathways towards activation of ERK1/2, JNK and AKT in schwannoma cells. Using various inhibitors SU6656 (Src), FTI-277 (Ras), Wortmannin (PI3K), GF109203x (PKC), previously validated in our cells,[10,12,25] we found that Gas6 stimulated ERK and JNK, recruiting Src and PI3K but not involving Ras and PKC (Figures 3g and h). Gas6-mediated AKT activation proceeded via Src and PI3K but not via Ras and PKC (Figure 3i). Thus the ERK, JNK and AKT pathways share similar mediators such as Src and PI3K.

Axl triggers Gas6-mediated activation of Src and FAK but not ERK, JNK or AKT. As Gas6 is an agonist for all TAM family receptors and Gas6-induced proliferation, and cell-matrix adhesion can be blocked by Axl knockdown, we used Axl shRNA to monitor which signalling pathways are triggered by this particular receptor. We demonstrate that Axl shRNA decreased Gas6-mediated Src and FAK (Figures 4d–f) but not ERK1/2, JNK or AKT activity (Figures 4a–c). These data therefore suggests that Gas6-mediated activation of ERK1/2, JNK and AKT (as shown in Figures 3a–c) probably proceeds via other TAM family receptors overexpressed in schwannoma such as Tyro3 and Mer.

NFκB is upregulated in schwannoma

We observed that Axl receptor activation leads to downstream activation of Src and FAK, but not of ERK1/2, JNK or AKT upon stimulation with Gas6. However, Axl activation by Gas6 does increase schwannoma proliferation and cell-matrix adhesion. We therefore hypothesized that an additional pathway must be involved in Gas6/Axl-mediated signalling towards increased proliferation and cell-matrix adhesion. The transcription factor NFκB acts downstream of Axl in cell lines and its activity is regulated by Gas6.[28] Moreover, NFκB regulates expression of FAK,[29] which localizes to the nucleus in schwannoma cells leading to increased proliferation and cell-matrix adhesion.[12] NFκB is known to be an anti-apoptotic factor in Schwann cells.[30] We therefore investigated the expression and signalling of NFκB in human schwannoma downstream of Axl.

We demonstrate that NFκB (p65 subunit) is strongly overexpressed in schwannoma cells (Figure 5a) owing to merlin deficiency as merlin reintroduction into primary human schwannoma cells decreased NFκB (p65) expression (Figure 4b). Immunocytochemistry showed nuclear localization of NFκB in both Schwann and schwannoma cells (Figure 5c) as expected. The levels of phosphorylated NFκB (P-Ser529), which increases NFκB transcriptional activity was also elevated in schwannoma cells (Figure 5d).

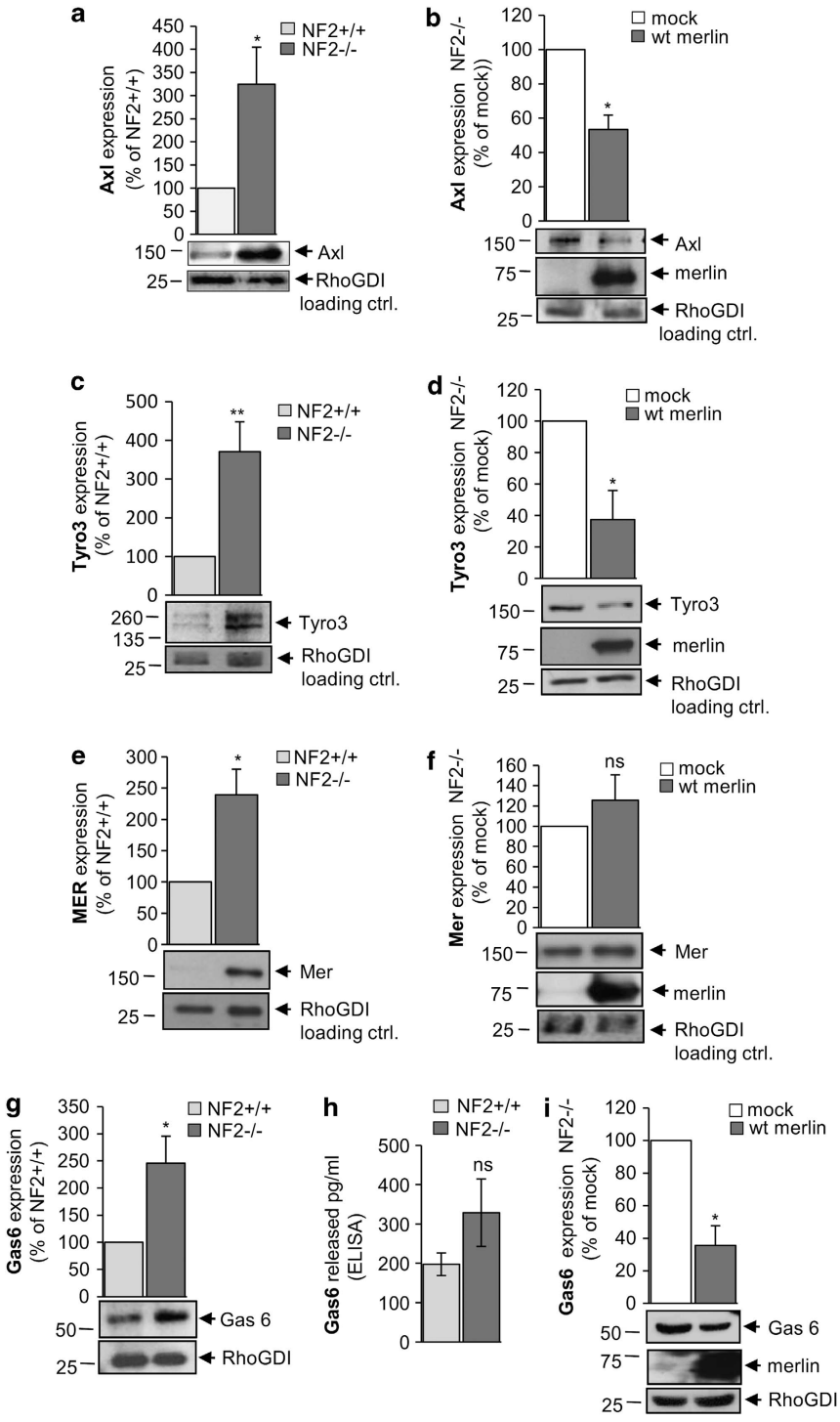

Figure 1. TAM family receptors are overexpressed in schwannoma. Human primary schwannoma cells (NF2 − / −) display increased expression of Axl ($n = 7$) (**a**), Tyro3 ($n = 5$) (**c**) and Mer ($n = 3$) (**e**) compared with normal schwann cells (NF2 + / +). Axl ($n = 5$) and Tyro3 ($n = 3$) expression is dependent (**b**, **d**) and Mer ($n = 3$) independent on merlin (**f**). TAM family receptors' agonist Gas6 is overexpressed in schwannoma (NF2 − / −) detected by western blotting in cells ($n = 4$) (**g**). Gas6 is released from both Schwann (NF2 + / +) and schwannoma cells (NF2 − / −) detected by enzyme-linked immunosorbent assay (ELISA) (NF2 + / + , $n = 3$, NF2 − / − , $n = 6$) (**h**). Gas6 expression is merlin-dependent ($n = 4$) (**i**). For receptor expression, the cells were cultured, lysed and detection was performed by western blotting. In **a**, **c**, **e** and **g**, the data are normalized to normal Schwann cells (NF2 + / +). In **b**, **d**, **f** and **i,** data are normalized to mock-infected cells (mock). In **b**, **d**, **f**, and **i**, the cells were infected with either mock (white bars) or wild-type (wt) (grey bars) merlin-containing adenovirus. *N* represents the number of samples from different patients (*$P < 0.05$, **$P < 0.01$). RhoGDI is used as a loading control as previously described in Hanemann *et al.*[52]

NFκB (p65 subunit) activity was potentiated by Gas6 (40 ng/ml) from 5 min up to 5-h stimulation, as shown by western blotting (Figure 5e) and by luciferase reporter gene assay (Figure 5f). By 24 h after stimulation with Gas6 (40 ng/ml), NFκB activity had decreased (Figures 5f and g). Axl shRNA decreased the levels of phosphorylated/active (Gas6 40 ng/ml, 5 min) NFκB (p65 subunit) (Figure 5h).

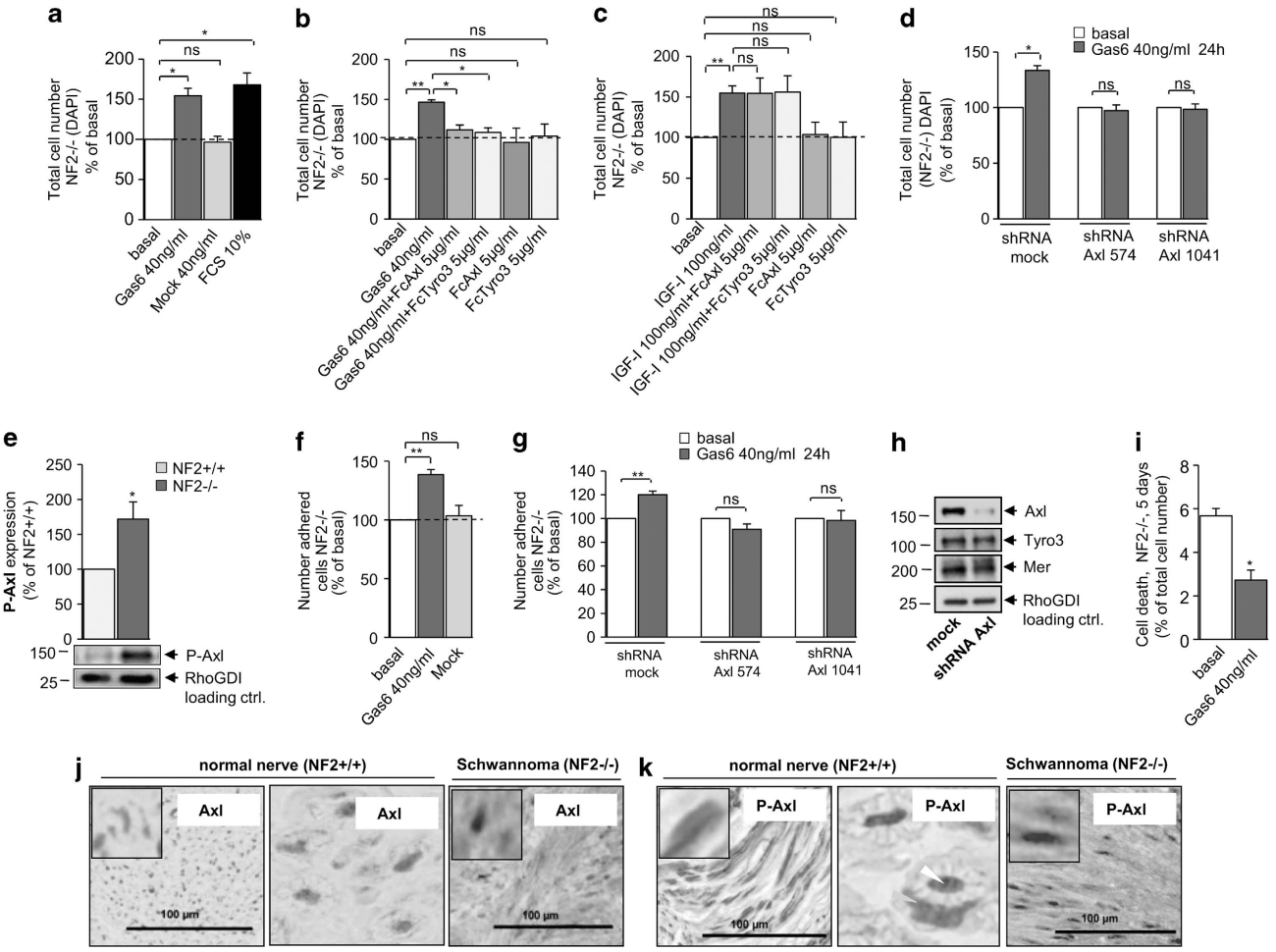

Figure 2. The role of Gas6 in schwannoma proliferation, cell-matrix adhesion, survival and activation of Axl receptor. Gas6 increases schwannoma basal proliferation in the same range as 10% FCS, mock medium (negative control for Gas6) had no effect on proliferation ($n = 6$) (**a**). Gas6-mediated proliferation is specific and inhibited by FcAxl and FcTyro3 chimeras ($n = 3$) (**b**) meanwhile, IGF-I-mediated proliferation is unaffected ($n = 3$) (**c**). Gas6-mediated proliferation is Axl dependent (**d**) shown using Axl shRNA (constructs 574 and 1041) ($n = 3$). Gas6 potentiates phosphorylation/activity of Axl ($n = 6$) (**e**). Gas6-mediated cell-matrix adhesion is increased by Gas6 but not mock control ($n = 8$) (**f**), which is Axl dependent ($n = 3$) (**g**). Axl shRNA is specific downregulating only Axl but not Tyro3 and Mer receptors ($n = 3$) (**h**). Schwannoma cell viability is potentiated by Gas6 (i). DAPI staining was used for proliferation monitoring, DAPI and propidium iodide for survival. Axl **(j)** and Phospho-Axl (P-Axl) (**k**) are strongly expressed in schwannoma tissues (NF2 − / − ; $n = 5$) compared with normal tissues (NF2 + / + ; $n = 5$) shown by immunohistochemistry in human tissues. Strong nuclear and cytoplasmic immunoreactivity is seen in schwannoma cells (**j** and **k**). In normal nerve tissue (**k**), P-Axl staining is seen in the axon (white arrow head) and paranuclear Schwann cell cytoplasm (yellow arrow head). *N* represents number of samples from different patients (*$P < 0.05$, **$P < 0.01$). RhoGDI is used as a loading control as previously described in Hanemann *et al.*[52]

The expression of survivin, cyclin D1 and FAK are potentiated by Gas6 in a NFκB-mediated manner in schwannoma cells. As both Gas6 and NFκB are known to regulate the survival and proliferation of Schwann cells[30] and are linked to cyclin D1 and FAK expression, which are relevant for tumour development,[29,31] we investigated the role of the Gas6/NFκB signalling axis in the expression of survivin, cyclin D1, and FAK.

Survivin. Survivin was overexpressed in primary human schwannoma cells (Figure 6a). Gas6 (40 ng/ml, 24 h) potentiated the expression of survivin (Figure 6b) in a merlin-dependent manner (Supplementary Figure 2A).[11] NFκB was found to be involved in this process as both the NFκB inhibitor SN50 and NFκB shRNA (Supplementary Figure 1) significantly inhibited the Gas6-mediated expression of survivin (Figures 6c and d). These data suggest that the Gas6/Axl/NFκB signalling axis may have a role in the pathological survival of schwannoma cells.

Cyclin D1. Gas6 (40 ng/ml, 24 h) potentiated the expression of cyclin D1 (Figure 6e), which is overexpressed in schwannoma cells[32] in a merlin-dependent manner (Supplementary Figure 2B). NFκB acted as a mediator in the potentiation of cyclin D1 expression (Figures 6f and g). These data therefore suggest that the Gas6/Axl/NFκB signalling axis may have a role the pathological proliferation of schwannoma cells.

FAK. The expression of FAK was increased by Gas6 (40 ng/ml, 24 h) (Figure 6h). Reintroduction of merlin decreased FAK expression (Supplementary Figure 2C). The inhibition of NFκB by SN50 and NFκB shRNA (Supplementary Figure 1) decreased the Gas6-mediated increase of FAK expression (Figures 6i and j). These data therefore suggest that the Gas6/Axl/NFκB signalling axis regulates the expression of FAK in schwannoma. Furthermore, we also show in Figures 3d and e and Figures 4e and f that FAK activity is potentiated by Gas6/Axl signalling.

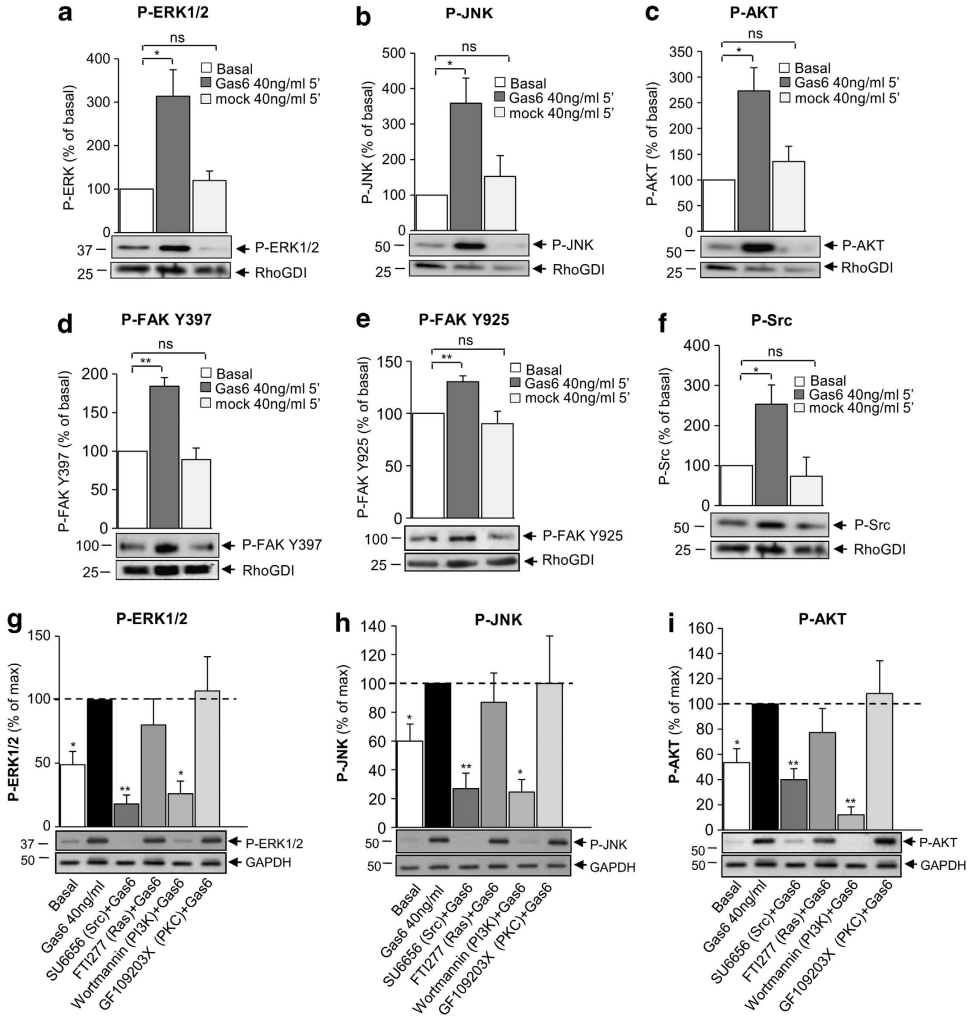

Figure 3. Dissection of signalling pathways activated by Gas6 in schwannoma cells. Schwannoma cell stimulation with Gas6 (40 ng/ml, 5 min) leads to increased ERK1/2 ($n=5$) (**a**), JNK1/2 ($n=4$) (**b**), AKT ($n=5$) (**c**), FAK (**d** ($n=3$) and **e** ($n=3$)) and Src ($n=6$) (**f**) phosphorylation/activation. The ERK1/2 ($n=7$) (**g**) and the JNK ($n=7$) (**h**) pathways follow similar route recruiting PI3K and Src and by passing Ras and PKC. The AKT pathway proceeds also via PI3K and Src ($n=7$) (**i**). In **a**–**i**, the cells were serum starved for 24 h and then stimulated for 5 min with Gas6 (40 ng/ml). In **g**–**i**, cells were pre-treated with different inhibitors SU6656 (Src, 1h), FTI277 (Ras, 24 h), wortmannin (PI3K 1 h) and GF109203x (PKC, 1 h). The results in **a**–**f** are normalized to the basal (non-stimulated) levels and in **g**–**i** to max response to Gas6 (40 ng/ml, 5 min). *N* represents the number of samples from different patients (*$P<0.05$, **$P<0.01$). RhoGDI is used as a loading control as previously described in Hanemann *et al.*[52]

Gas6/Axl-mediated proliferation of schwannoma cells depends on NFκB

We observed that NFκB is downstream of Axl regulating the expression of cell cycle protein cyclin D1 and also FAK in human schwannoma. Previously we have shown that FAK localizes to the nucleus and, together with Src, is responsible for integrin β1/IGFBP-1-mediated proliferation independently of ERK1/2 and JNK.[12] We therefore investigated here the role of NFκB in Gas6/Axl-mediated proliferation.

Both the NFκB inhibitor SN50 and shRNA for NFκB (p65 subunit) (Supplementary Figure 1) significantly decreased Gas6-mediated proliferation of schwannoma cells to basal levels (Figures 7a left panel and b) in parallel to preventing Gas6-induced increases in cyclin D1 (Figures 6f and g) and FAK (Figures 6i and j) expression. Schwannoma cell viability upon SN50 treatment for 24 h was not affected (Figure 7a right panel).

The role of NFκB in Gas6/Axl-mediated cell proliferation, cell-matrix adhesion and survival in schwannoma cells

NFκB is specifically involved in Gas6/Axl-mediated proliferation in schwannoma, which converges with integrin β1 signalling. To test whether NFκB is specific for Gas6-mediated proliferation, we also investigated its role in PDGF-DD-, IGF-I- and β-heregulin-mediated proliferation, all known to be relevant for schwannoma pathology.[10,11,26] We observed that NFκB is specific or selective for Gas6 signalling towards schwannoma proliferation, as SN50 had no effect on either PDGF-DD or IGF-I and β-heregulin-mediated proliferation (Figure 7c). Signalling triggered by IGFBP-1, acting via integrin β1, also utilized NFκB for proliferation (Figure 7c). Moreover, both IGFBP-1 (100 ng/ml) and Gas6 (40 ng/ml) increased the expression of NFκB after 24 h of stimulation (Figure 7d). These data suggest a degree of convergence between Gas6/Axl and integrin β1/IGFBP-1 pathways towards increased proliferation of schwannoma cells via NFκB.

NFκB mediates Gas6/Axl-potentiated cell-matrix adhesion and survival of schwannoma cells. We found that similarly to proliferation, Gas6/Axl-mediated cell-matrix adhesion and survival also occurred via recruiting NFκB, as observed because of the blockade of Gas6-mediated increase in cell-matrix adhesion by the NFκB inhibitor SN50 and NFκB (p65 subunit) shRNA (Figures 7e and f; Supplementary Figure 1) and decrease of survival by the

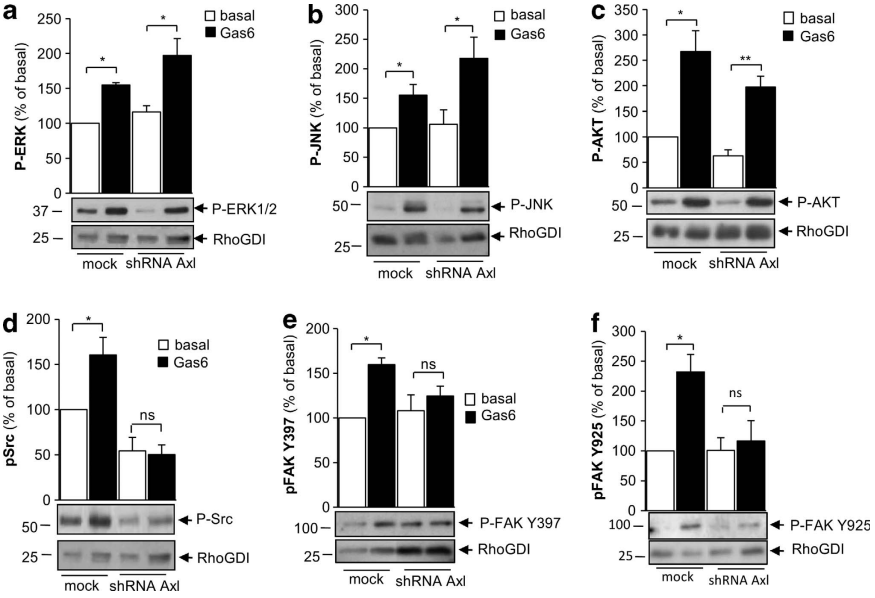

Figure 4. Signalling pathways activated specifically by Axl. Axl shRNA did not affect Gas6- mediated ERK1/2 ($n = 5$) (**a**), JNK (**b**) or AKT ($n = 5$) (**c**) activity but significantly decreased Gas6-mediated Src ($n = 6$) (**d**) and FAK (**e**) ($n = 6$) and (**f**) ($n = 5$) activity. The cells were infected by lentivirus-containing AxlshRNA, following that the cells were selected for infection, serum starved for 24 h and stimulated with Gas6 (40 ng/ml) for 5 min. The data were normalized to the basal mock infection levels. Statistics was performed comparing nonstimulated levels (white bars) with Gas6-stimulated levels (black bars). *N* represents the number of samples from different patients (*$P < 0.05$, **$P < 0.01$). RhoGDI is used as a loading control as previously described in Hanemann *et al.*[52]

NFκB inhibitor SN50 (Figure 7g). To study the survival of schwannoma cells is difficult as these cells survive, when cultured under growth factor withdrawal conditions, for weeks displaying only very minor decrease in cell viability in contrast to Schwann cells as shown in Figures 2i and 7g and previously published in Utermark *et al.*[22]

DISCUSSION

In this manuscript, we have investigated the role of Axl receptor signalling in the development of merlin-deficient schwannoma. We formulated our hypothesis that Axl may contribute to schwannoma development on the basis of previous findings: (1) positive regulation of Axl by E3 ubiquitin ligase CRL4DCAF1, which we and others have shown to contribute to schwannoma tumour growth;[19] (2) Axl interaction with oncogene Yes-associated protein, a member of Hippo pathway, which is under merlin regulation in schwannoma and is involved in increased proliferation of merlin-negative meningioma and mesothelioma;[20] (3) negative regulation of Axl by merlin; (4) the previously reported mitogenic effect of Gas6 on human Schwann cells acting via Axl.[21]

Our results demonstrate strong overexpression of TAM family receptors in human primary schwannoma cells compared with normal Schwann cells, supporting previous array data.[11] Strong immunostaining of Axl and active/phospho-Axl was also observed in schwannoma biopsies, within both tumour nuclei and the cytoplasm, compared with normal nerve tissues. Overexpression of Axl and Tyro3 is merlin-dependent in human primary schwannoma cells, which supports with previous data.[19] Interestingly, the overexpression of Mer was not dependent on merlin, suggesting a possibility of the involvement of additional merlin-independent mechanisms/mutations. Merlin-dependent overexpression of the TAM receptors agonist Gas6 was also observed in schwannoma cell cultures. Gas6 is released from both normal Schwann and schwannoma cells with slightly higher but nonsignificant levels in schwannoma, suggesting the possibility for autocrine or paracrine signalling. The impact of the TAM receptor/Gas6 signalling axis on tumourigenesis and schwannoma pathobiology was ascertained by functional assays, which demonstrated strong mitogenic and pro-adhesive effects of Gas6 on primary human schwannoma cells. Gas6 stimulation increased both proliferation and cell-matrix adhesion and had a pro-survival effect on schwannoma cells acting via the Axl receptor, which was previously observed in human Schwann cells[21] and other tumours.[33] Even though Gas6, known to be an agonist for Axl, Tyro3 and Mer, activates ERK1/2, JNK, AKT, FAK and Src in schwannoma, Gas6/Axl receptor-dependent signalling employed only FAK and Src. We thus suggest the presence of signalling specificity among TAM family receptors in schwannoma. As Axl mediates both increased proliferation and cell-matrix adhesion, but does not stimulate activation of pro-proliferative ERK1/2 and JNK and cell-matrix adhesion-stimulating ERK1/2,[10] we hypothesized that some other pathway must be involved in Axl-mediated proliferation, cell-matrix adhesion and survival. NFκB seemed to be a good candidate as it acts downstream of Axl in cell lines,[28] regulates expression of FAK which localizes to the nucleus in schwannoma cells leading to increased proliferation and cell-matrix adhesion,[12,29] its activity is positively regulated by Gas6 in NIH 3T3 (mouse fibroblast cells)[28] and negatively regulated by merlin in cell lines,[34] is pro-survival in mouse Schwann cells,[30] mitogenic via cyclin D1 and is overexpressed and constitutively active in tumours.[35] Here, we demonstrate a strong overexpression of NFκB in schwannoma cells, which is merlin dependent. These data are supported by previous studies by Kim *et al.*,[36] showing that in NIH3T3 and C6 (rat astroglioma cells) cells, NFκB expression is also merlin-dependent. In addition, we found that transcriptional activity of NFκB was highly increased in schwannoma as shown by western blotting and reporter gene assays, and which was both Gas6- and Axl-dependent, suggesting NFκB as an important signalling molecule downstream of Gas6/Axl in schwannoma. The importance of NFκB in schwannoma is underlined by the discovery of its involvement in increased expression of pro-survival protein survivin,[11] shown to be overexpressed in schwannoma due to Gas6 stimulation. In addition, NFκB was found to be recruited in the Gas6-mediated increase in expression of cyclin D1, previously shown in our

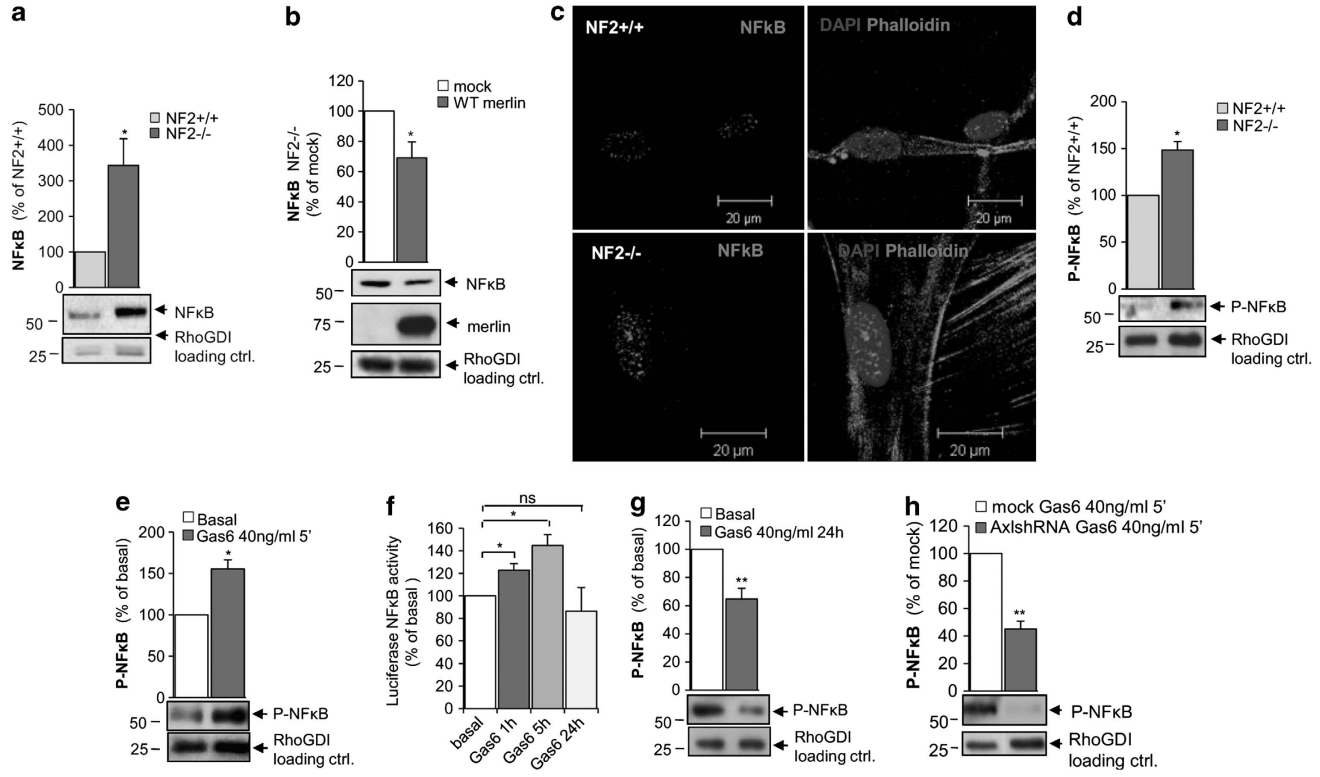

Figure 5. The role of NFκB in schwannoma. NFκB (p65 subunit) is overexpressed in schwannoma cells (NF2 − / −) compared with normal Schwann cells (NF2 + / +) ($n = 8$) (**a**) in merlin-dependent manner ($n = 6$) (**b**). NFκB (p65 subunit) localizes to the nucleus in both Schwann (NF2 + / +) and schwannoma cells (NF2 − / −) ($n = 3$) (**c**, immunocytochemistry). Phosphorylated/active NFκB (p65 subunit) is overexpressed in schwannoma cells ($n = 3$) (**d**). Gas6 potentiates schwannoma basal expression of phosphorylated/active NFκB ($n = 6$) (**e**, p65 subunit, western blotting) and NFκB transcriptional activity ($n = 3$) (**f**, reporter gene assay) upon stimulation for 5 min, 1 and 5 h, respectively. The levels of transcriptionally active NFκB (**f**) and of phosphorylated/active NFκB (p65 subunit) ($n = 3$) (**g**) are decreased to the basal levels after 24 h of stimulation with Gas6. The increased phosphorylation/activity of NFκB (p65 subunit) (Gas6 40 ng/ml, 5 min) in schwannoma is Axl dependent ($n = 3$) (**h**). In (**b**), the cells were infected with either mock (white bar) or wild-type (wt) merlin (grey bar) containing adenovirus. In H the cells were infected with mock or Axl shRNA lentivirus. The data in B and H are normalized to mock (white bars). In An and D the data are normalized to normal Schwann cells (NF2 + / +), in E, F and G to basal (non-stimulated, white bars) levels. *N* represents number of samples from different patients (*$P < 0.05$, **$P < 0.01$). RhoGDI is used as a loading control as previously described in Hanemann *et al.*[52]

laboratory to be overexpressed in schwannoma.[37] Our previous work showed the overexpression of FAK in schwannoma[10] and its localization to the nucleus and focal adhesions increases the proliferation and cell-matrix adhesion of schwannoma cells upon IGFBP1-mediated integrin β1 activation independently of ERK1/2 and JNK.[12] Here we revealed that FAK activity is potentiated by Gas6, which is mediated by Axl receptor (5-minutes stimulation). FAK expression is also strongly potentiated by Gas6 but mediated by NFκB (24 h of stimulation). NFκB is involved in the increased schwannoma proliferation and cell-matrix adhesion, sharing the same pathway as IGFBP1/integrin β1, as NFκB inhibition decreased only Gas6 and IGFBP1 but not IGF-I/IGF-IR (insulin-like growth factor I receptor)-, PDGF-DD/PDGFR (platelet-derived growth factor receptor)- or heregulin/ErbB2/3-mediated proliferation. Also, IGFBP1 and Gas6 were able to potentiate the expression of NFκB after 24 h of stimulation. Thus, NFκB could be a very promising target in schwannoma and specific inhibitors targeting this transcription factor are under development.

In conclusion, we have shown, in this manuscript, that all members of TAM family, Axl, Tyro3 and Mer, are overexpressed in schwannoma. However, Axl do not act via ERK1/2, JNK or AKT in our model employing Src/FAK and NFκB instead. We have therefore in this paper focused on Axl as its signalling seemed to be unorthodox and matching our previous findings in schwannoma, which we could not fully explain before. In order to develop the most effective therapy, all receptors and signalling pathways should be investigated and tested, before decision on what to take forward into clinical trials.

On the basis of present and previous results, we suggest Axl and members of its downstream signalling cascade, including NFκB and Src, as potential therapeutic targets. Axl has previously been suggested as a biomarker for NF1-related tumours (Johansson *et al.* (2012), unpublished data) and here we also put forward Axl as a new candidate biomarker for spontaneous and NF-2-related schwannomas. Interestingly, soluble forms of TAM receptors have been detected in human plasma,[38,39] and therefore could be easily detected in patients for diagnostic purposes. In terms of therapeutics, there are different approaches to inhibit the Axl receptor and its signalling, including small-molecule inhibitors such as MP470, a novel c-Kit/AXL kinase inhibitor[40] or treatment with soluble Axl ectodomains that neutralize Gas6.[38] One of the new Axl inhibitors is already tested in clinical trials for NF1 tumours (Johansson *et al.* (2012), unpublished data), and thus could be re-profiled and tested, alone or in combination with other inhibitors, in schwannoma and other merlin-deficient tumours.

MATERIALS AND METHODS

Cell culture conditions

The primary human Schwann and schwannoma cells were obtained after patient's consent, as described elsewhere[14] and cultured in DMEM

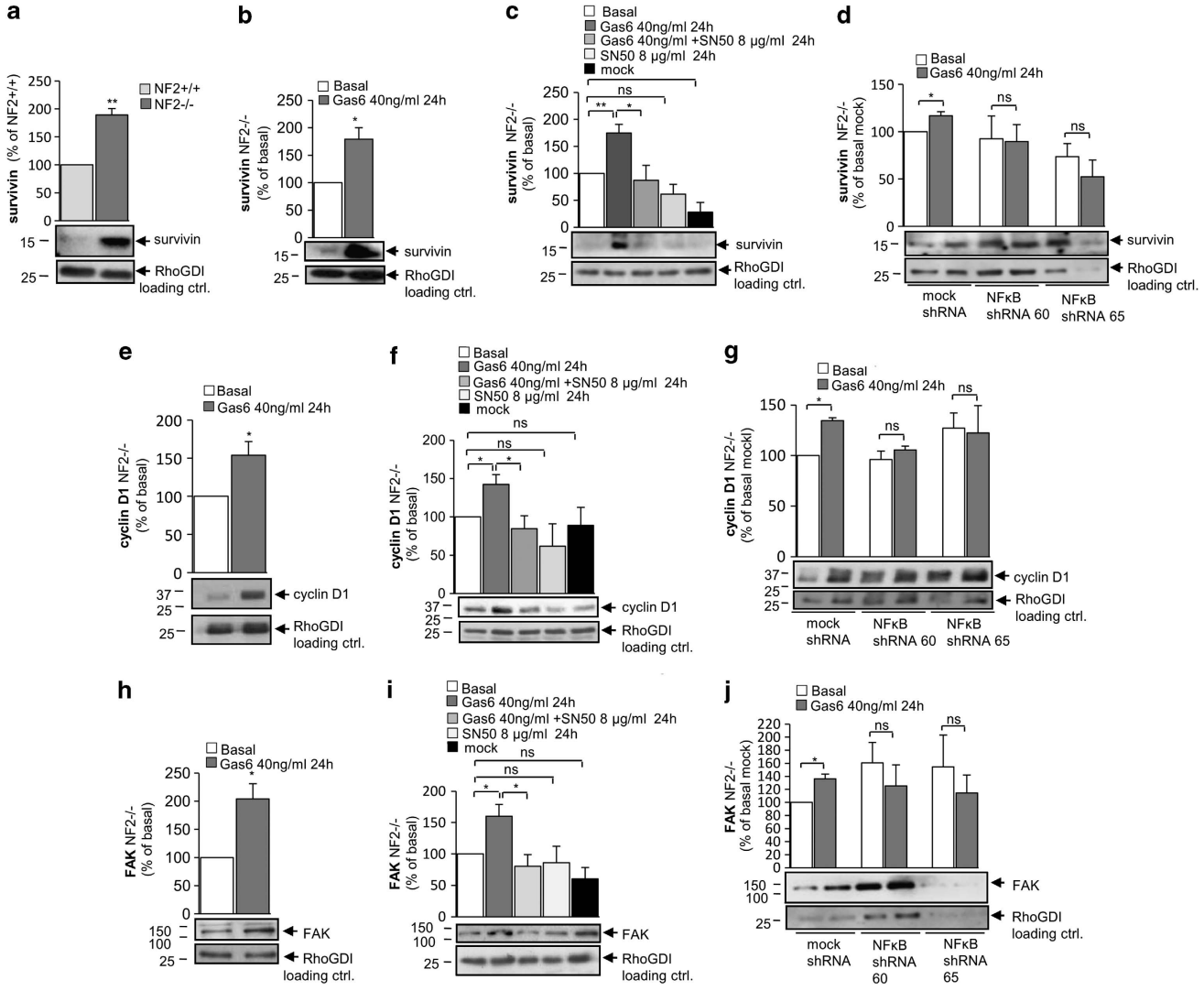

Figure 6. The role of NFκB in increased expression of Src, cyclin D1 and FAK in schwannoma. Pro-survival protein survivin is overexpressed in schwannoma (NF2 − / −) ($n = 4$) (**a**). Survivin ($n = 3$) (**b**), cyclin D1 ($n = 10$) (**e**) and FAK ($n = 6$) (**h**) expression is potentiated by Gas6 upon 24-h stimulation. Gas6-mediated increase in the expression of survivin (**c** ($n = 3$), **d** ($n = 3$)), cyclin D1 (**f** ($n = 6$), **g** ($n = 3$)) and FAK (**i** ($n = 6$), **j** ($n = 4$)) are dependent on NFκB. In (**a**) the data are normalized to normal Schwann cells (NF2 + / +), in **c**, **f** and **i** to the basal (nonstimulated) cells, in **d**, **g** and **j** to basal mock-infected cells. In **b**, **c**, **e**, **f**, **h** and **i** statistical comparisons were made between nonstimulated (basal, white bars) and stimulated (Gas6, grey bars). Both NFκB inhibitor SN50 (**c**, **f** and **i**) and shRNA (**d**, **g** and **j**) were used to inhibit NFκB-mediated signalling. *N* represents the number of samples from different patients (*$P < 0.05$, **$P < 0.01$). RhoGDI is used as a loading control as previously described in Hanemann *et al.*[52]

supplemented with 10% FCS, 0.5 µM forskolin, 10 nM β-heregulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 2.5 µg/ml insulin (complete medium, growth factor medium). In every experiment, minimum three samples from three different patients and donors were included. In every experiment, minimum three samples from three different patients and donors were included. For the activation of different signalling pathways, the cells were stimulated for 5 min as we previously showed that, in schwannoma, the maximum activation of signalling pathways is obtained upon short period of stimulation.[10] Proliferation assays and the increase of protein expression were performed upon 24-h post-stimulation.

Inhibitors and chemicals

SU6656 and FTI-277 were from Calbiochem (La Jolla, CA, USA), GF109203x from Tocris Bioscience (Bristol, UK), SP600125 from Affiniti Research Products, Ltd. (Exeter, UK), Wortmannin from Tocris and SN50 from Calbiochem. Axl shRNA and NFκB shRNA were from Open Biosystems (Huntsville, AL, USA). DAPI and propidium iodide were from Sigma (St Louis, MO, USA) and U0126[41,42] from Promega (Madison, WI, USA). Human Gas6 was a gift from Dr Sassan Hafisi (Portsmouth University, UK). Recombinant Axl Fc Chimera and Dtk (Sky) Fc Chimera were from R&D Systems (Minneapolis, MN, USA). Most of these drugs have been previously tested in our human primary schwannoma model.[10,11,27,43] The concentrations of inhibitors have been previously confirmed by others.[41,42,44–49]

shRNA knockdown

GIPZ-shRNAmir lentiviral particles encoding a short-hairpin RNA (shRNA) with nonsilencing sequence (mock) or sequences targeting specific genes were used. Infections were performed, as previously described in Ammoun *et al.*[12,16]

Sequences of Axl shRNA lentivirus. TRCN0000000574: 5′-CCGGCGAAAGAAGGAGACCCGTTATCTCGAGATAACGGGTCTCCTTCTTTCGTTTTT-3′
TRCN0000001041: 5′-CCGGGCTGTGAAGACGATGAAGATTCTCGAGAATCTTCATCGTCTTCACAGCTTTTT-3′

Sequences of NFκB shRNA lentivirus. V3LHS_633760: 5′-TGCTGTTGACAGTGAGCGACAAGCTGATGTGCACCGACAATAGTGAAGCCACAGATGTATTGTCGGTGCACATCAGCTTGCTGCCTACTGCCTCGGA-3′

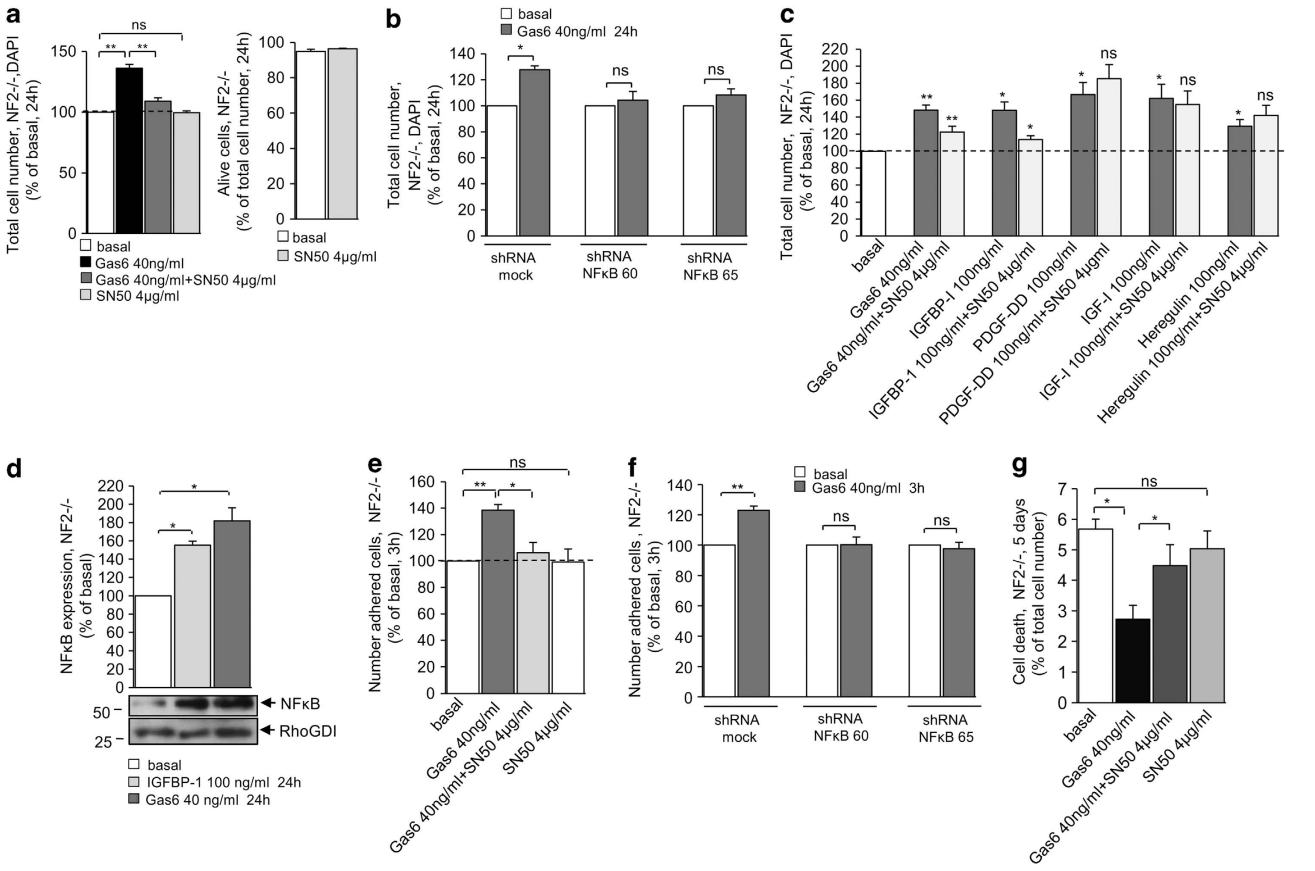

Figure 7. The role of NFκB in Gas6-mediated proliferation, cell-matrix adhesion and survival. Gas6- mediated proliferation is NFκB dependent (**a** left panel and **b**), monitored using both NFκB inhibitor SN50 ($n = 6$) (**a**) and shRNA (constructs shRNA NFκB60 and shRNA NFκB65) (**b**). SN50 was not toxic for the cells upon 24-h incubation (**a** right panel). NFκB inhibitor SN50 inhibited only Gas6 and IGFBP1/integrin β1-mediated proliferation but not PDGF-DD, IGF-I or heregulin-mediated proliferation (**c**). Both Gas6 and IGFBP1 potentiate the expression of NFκB after 24-h stimulation (**d**). Gas6-mediated cell-matrix adhesion is NFκB dependent (**e**, using NFκB inhibitor SN50) and (**f**, using shRNA NFκB constructs 60 and 65). Gas6 potentiates schwannoma cell survival in NFκB-dependent manner (**g**). In **a**, **c**, **d** and **e** the data are normalized to the basal (nonstimulated, white bars) cells, in **b** and **f** to basal nonstimulated infected cells (white bars). *N* represents the number of samples from different patients (*$P < 0.05$, **$P < 0.01$).

V2LHS_98065: 5′-TGCTGTTGACAGTGAGCGCGGGAATCCAGTGTGTGAAG AATAGTGAAGCCACAGATGTATTCTTCACACACTGGATTCCCATGCCTACTGCC TCGGA-3′

V2LHS_98066: 5′-TGCTGTTGACAGTGAGCGCGCTCAGTGAGCCCATGGAA TTTAGTGAAGCCACAGATGTAAATTCCATGGGCTCACTGAGCTTGCCTACTGCC TCGGA-3′

Re-introduction of merlin

Mock (control adenovirus containing a GFP) and merlin wild-type (recombinant adenovirus AdNF2) were gift from J Testa.[50] Cells were treated with virus for 24 h, followed by incubation in fresh growth factor medium for additional 24 h.

Immunoblot analysis

Following electrophoresis and western blotting,[23] detection of proteins was performed using anti-Axl, anti P-Axl (Y779), anti-Tyro3 and anti-Gas6 (R&D Systems), anti-Mer (Abcam, Cambridge, UK), anti-NFkappaBp65, anti-P-Src (Y416), anti- survivin, anti-cyclin D1 and anti-FAK (Cell Signalling, Danvers, MA, USA), anti-integrin β1 and anti-phospho NFκB p65 Ser529 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-active-MAPK (anti-pThr(183)-pTyr (185)-ERK1/2) (Promega), anti-phospho-AKT (Ser473) (Cell Signaling), anti-phospho-FAK (Tyr925) (Cell Signaling), anti-phospho-FAK (Tyr397) (Chemicon International, Temecula, CA), anti-phospho-JNK (Thr183/Tyr185) (Cell Signaling), and HRP-conjugated secondary antibodies (Bio-Rad, Hercules, CA, USA). ECL-plus (Amersham, Buckinghamshire, UK) was used as a substrate for HRP. As short-term stimulations do not affect protein expression levels,[51] we have used a generic loading controls Rho-GDI previously established for our system (Anti-RhoGDI antibody; Santa Cruz Biotechnology)[52] and GAPH (Millipore, Watford, UK). Images were scanned and processed using Corel Paint Shop Pro Photo XI software (Corel, Ottawa, ON, Canada). Images represent the original data.

Immunohistochemistry

Immunohistochemistry was performed as previously described in Ammoun *et al.*[12,53] The sections were pre-treated with EDTA buffer at pH 9 microwave for 10–30 min, before overnight incubation with the primary antibodies at dilutions 1:75 (Axl) and 1:100 (pAxl). Detection was performed with the Vectastain Universal Elite ABC kit (Vector Laboratories, Peterborough, UK). Controls were by omission of the primary antibody. Anti-Axl was from Abnova (Taipei, Taiwan) and anti-phospho Axl from R&D Systems. Tissue from five schwannomas and five controls (normal and reactive nerve) were used.

Cell proliferation and viability assays

Cells were cultured for 24 h in DMEM alone, DMEM containing 40 ng/ml Gas6 (from conditioned medium collected form Gas6 producing HEK293 cells) or mock (from conditioned medium collected from equivalent, non-Gas6-producing HEK293)[54] or growth factor medium, as described in Ammoun *et al.*[10,12] Inhibitors pre-incubation was performed, as described in Ammoun *et al.*[12] Cell count and DAPI, PI staining, as previously described Ammoun *et al.*[10,12]

Adhesion assay

Adhesion assay was performed, as previously described in Utermark *et al.*[17] Cells were stimulated with 40 ng/ml Gas6 (medium collected form Gas6

producing cells) or mock (medium collected from cells unable produce Gas6) for 3 h. Inhibitors were added 40 min before stimulation.

Luciferase reporter assay

The cells were seeded onto 96-well pre-coated plates.[14] The day after the cells were infected with ready-to-transduce lentiviral particles expressing either an inducible transcription factor-responsive reporter gene (firefly luciferase) or expressing firefly luciferase without any additional transcriptional response elements (negative control) or constitutively expressing firefly luciferase (positive control), as described by the manufacturer (SABiosciences Corporation, Quiagen company; MD, USA). On day 3, medium containing Cignal lentiviral particles was replaced with fresh GFM (growth factor medium) culture medium. Day 4, medium was changed to serum-free DMEM alone or containing 40 ng/ml Gas-6. Detection was performed using Firefly Luciferase Assay System (Promega), as described by the manufacturer.

Data analysis

For statistical analysis student's two-tailed *t*-tests and ANOVA were used. Experiments were performed in at least triplicates using at least three independent batches of cells from different individuals. ns (not significant): $P > 0.05$; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. In figures, mean ± s.e.m. is given.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)