Molecular and mechanical functions of the intermediate filament protein GFAP
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Citation for published version (APA):
Stassen, O. M. J. A. (2016). Molecular and mechanical functions of the intermediate filament protein GFAP: Beyond the wickerwork

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Chapter 7

Summary and Discussion
Chapter 7

Summary

Astrocytes are brain cells that are essential for the brain’s physiological homeostasis and they also respond strongly to a variety of pathologies that affect this homeostasis (Sofroniew and Vinters, 2010). Glioma or astrocytoma are tumours originating from deregulated astrocytes, glia progenitors or neural stem cells (Chen et al., 2012). All cells in the body have a cytoskeleton important for cell shape, motility, scaffolding and signalling processes. A hallmark of the cytoskeleton of astrocytes and glioma cells is that it contains the intermediate filament (IF) protein Glial Fibrillary Acidic Protein (GFAP). The production of GFAP is increased in high quantities in neuropathology. The gene expression of GFAP is tightly regulated in both quantity and splicing during development. Splicing of the GFAP mRNA can generate at least 10 different isoforms, of which GFAPα and GFAPδ are the most prominent isoforms in the brain. GFAPδ is enriched in the neurogenic niche and might therefore also be related to the formation of glioma. Although GFAP is already known since 1970 (Eng et al., 2000), the function of this protein and its later identified isoforms remains an open question. In this thesis we have investigated cell biological, molecular biological, and biophysical aspects of the IF network, in particular of GFAP, in astrocyte and astrocytoma biology.

In Chapter 1 we introduced IFs, more specifically type III IFs, and compared the molecular interactions and biological significance of GFAP with those of Vimentin (Vim). These proteins have a similar structure and are closely related proteins, although the N-terminal head and C-terminal tail domain of GFAP and Vim differ substantially. Despite the low degree of similarity between GFAP and Vim in the head and tail, these IF proteins do appear to be involved in similar global processes in the cell. The head and tail domains are functionally important as these domains interact with other proteins, and their high serine and threonine content make them targets for post-translational modifications. Therefore, it is likely that the two proteins have distinct specific molecular functions within astrocytes.

In Chapter 2 we induced reactive gliosis in an astrocyte cell line (astrocytoma) and in primary human astrocytes, and investigated the changes in whole genome gene expression profiles by a transcriptomics platform. To induce reactive gliosis we used a mechanical injury device that inflicted a short-lasting but severe stretch of the substrate the cells were cultured on. This approach mimics the type of injury the cells would be exposed to in traumatic brain injury. Curiously, both the astrocytoma and the primary cells used in this mechanical injury model did not reveal major changes at a transcriptional level. Several potential causes for this lack in response were investigated by changing the injury protocol. In contrast to the reported
findings of other studies, we were unable to induce astrocytosis, evidenced by GFAP increase or other related genes, by our mechanical stretching protocol, implying a strong resilience of these cultured cells to stretching injury.

In Chapter 3 we determined whether different GFAP isoforms have an effect on the IF dynamics in cultured cells. We observed that the turnover rate between the soluble and filamentous pool of GFAPδ is lower than that of GFAPα. For both isoforms, a higher expression resulted in larger focal adhesions, although this did not affect cell motility. High expression of GFAPδ resulted in a change of cell shape and induced a collapse of the complete IF network but, remarkably, without further apparent effects on cell motility and proliferation.

In Chapter 4 we investigated the role of GFAP in astrocytoma biology. So far, conflicting reports have been published on the relation between GFAP and astrocytoma malignancy. We first analysed the expression of the different isoforms in the Cancer Genome Atlas (TCGA) database. We showed that GFAPα mRNA...
expression declines with increasing malignancy while GFAPδ expression remains at a constant expression level. Together this results in an increase in the GFAPδ/α ratio, which could reflect a measure of stemness, since GFAPδ is highly expressed in neural stem cells. To determine the downstream changes in gene expression induced by an increase in GFAPδ/α ratio, we modulated the GFAP isoform content in different astrocytoma cell lines. By microarray transcriptome analysis we found that changing the GFAPδ/α ratio changes the expression of genes relevant for direct and indirect cell interactions with the external environment, including the extracellular matrix and the interaction with other cells. Projecting this in vitro dataset on the TCGA patient data revealed 8 target genes that are involved in tumour malignancy and are regulated by the GFAP isoform ratio. Most importantly, the expression of these genes is prognostic for patient outcome.

In Chapter 5 we analysed mRNA transcript profiles of acutely isolated astrocytes and microglia from GFAPko and VIM-GFAPko mice. Similar to the findings in Chapter 4 it became clear that GFAP and Vim are both involved in cell-environment interactions. For Vim, an added role in angiogenesis emerged as an overrepresented ontology. The analysis of microglia, the brain’s innate immune cells, in the GFAPko revealed that prominent transcriptional changes take place in these cells induced by GFAPko in the astrocytes. This supports the finding of a function of GFAP in cell-environment interaction. We generated gene clusters based on their expression patterns and identified genes regulated by GFAP, Vim, or both GFAP and Vim. This approach revealed multiple mRNA downstream targets of GFAP and Vim, as well as transcripts regulated by the modulation of only one of the two IF proteins, implying both distinct and shared pathways downstream of GFAP and Vim.

In Chapter 6 we investigated the contribution of GFAPα and GFAPδ to cell mechanics, a well-established function of IFs. Using skeletonized IF networks stripped from other cytoskeletal components, we were able to probe the pure mechanical contribution of GFAP isoforms to IF network mechanics. This was done by the multiple particle tracking microrheology method, which revealed that the increase of both GFAP isoforms had a stiffening effect on the network.

In conclusion, we found evidence that GFAP is involved in the communication between cells or between cells and the extracellular matrix and that GFAP contributes to the mechanical properties of the cell (Fig. 1). In the following discussion I will focus on a few common themes emerging from the transcriptome analyses and make suggestions for further paths of investigation to take in order to narrow down molecular mechanisms that are regulated by GFAP and other IF proteins in astrocytes.
Emerging common molecular mechanisms controlled by GFAP

In Chapters 4 and 5 we modulated the compositions of the GFAP IFs and performed a transcriptomics analysis. Combining these data, that were gathered from *in vitro* in human cell lines and *in vivo* mouse astrocytes, revealed novel common molecular pathways that are regulated by the composition of the IF network.

First, we explored more in depth the consequences of modulating the GFAP network in astrocytoma cells in *vitro* with either a recombinant expression or a knockdown of GFAP isoforms. This analysis revealed several genes or molecular pathways that were either controlled by GFAP in general or by a change in the GFAPδ/α ratio shift. Then we searched for genes and molecular pathways that were regulated in both the human *in vitro* experiments as well as in the mouse astrocytes with different GFAP networks. The latter genes and pathways imply conserved actions induced by GFAP independent of the model system.

**GFAP isoform specific responses observed *in vitro***

In the *in vitro* modulation of the GFAP network in Chapter 4, we identified two main clusters in GFAP induced differentially expressed genes (DEGs; Fig. 2). The first cluster is associated with a change in GFAPδ/GFAPα ratio. Here, we observed similar changes in expression of genes in both the GFAPα+ and GFAPδ+ conditions (i.e. up or down in both conditions). This cluster was enriched for genes coding for growth factor...
receptors. We expected to find an opposite regulation in the GFAPα+ condition, but interestingly this was not the case (Fig. 2). A possible cause could be that GFAPδ is sequestered by GFAPα, and that the ratio-dependent genes respond particularly to high unsequestered GFAPδ. Under physiological conditions GFAPα is 10 times higher expressed than GFAPδ, so more GFAPα will not cause a drastic change in IF stoichiometry and GFAPδ sequestering. In contrast more GFAPδ will change the IF network and will lead to a collapse (Chapter 3, Fig. 1). In the GFAPδ+ and the GFAPα- condition another similarity is that the amount of GFAP that is close to the cell membrane is reduced, in the first case due to reduced total GFAPα, in the other case due to the collapse of the network (Chapter 4, Sup. Material 2). The IF network forms a scaffold for phosphatases and kinases, and therefore these enzymes can interact with different targets when the IF network is in close proximity of the cell membrane. In addition, modulation of GFAP in astrocytes, as well as different IFs in other cell types, results in mislocalization of membrane proteins, which may be dependent on the membrane proximity of the IF network (Alam et al., 2013; Hughes et al., 2004; Ivaska et al., 2005). The second cluster contains the GFAP-dependent genes (Fig. 2). These were either positively or negatively correlated to the level of

<table>
<thead>
<tr>
<th>GFAPδ/α isoform ratio dependent</th>
<th>Gene Function</th>
<th>GFAP dependent</th>
<th>Gene Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>KDR</td>
<td>GF signalling</td>
<td>AJAP1</td>
<td>Adherens Junction</td>
</tr>
<tr>
<td>BAIAP2L1</td>
<td>GF signalling</td>
<td>CRYAB</td>
<td>Heat Shock Protein</td>
</tr>
<tr>
<td>KIAA1217</td>
<td>Unknown</td>
<td>LEPREL1</td>
<td>ECM modifier</td>
</tr>
<tr>
<td>SLC1A3</td>
<td>Glu transporter</td>
<td>RAMP1</td>
<td>CGRP receptor processing</td>
</tr>
<tr>
<td>ACP5</td>
<td>ECM modifier</td>
<td>A2M</td>
<td>Peptidase</td>
</tr>
<tr>
<td>PRKZ</td>
<td>Kinase</td>
<td>AKR1C1</td>
<td>Progesterone metabolism</td>
</tr>
<tr>
<td>GGTLC1</td>
<td>Glutathione metabolism</td>
<td>TRIML2</td>
<td>P53 regulation</td>
</tr>
<tr>
<td>PLEKHS1 (C10orf81)</td>
<td>Pleckstrin domain</td>
<td>LAMA1</td>
<td>ECM protein</td>
</tr>
<tr>
<td>ERBB2</td>
<td>GF signalling</td>
<td>OGDHL</td>
<td>Oxoglutarate dehydrogenase</td>
</tr>
<tr>
<td>RNF150</td>
<td>Ubiquitination?</td>
<td>NRXN3</td>
<td>Axonal guidance</td>
</tr>
</tbody>
</table>

Table 1: Genes that correlate with either GFAPδ/α ratio or with total GFAP, based on Chapter 4. Genes written in bold correlate positively with GFAPδ/α or GFAP, whereas genes in italics correlate negatively. GF: Growth Factor; Glu: Glutamate; CGRP: Calcitonin-gene-related peptide. Gene functions based on Maglott et al., 2011.
GFAP expression, regardless of the isoform. Table 1 shows the 10 genes with the most significant changes in these two groups of DEGs, i.e. GFAPδ/α-ratio-dependent and GFAP-dependent genes.

A logical question is whether this list of genes also pops up in the astrocytoma samples from patients. In the analysis of the TCGA data (Chapter 4) we showed that GFAPα expression was significantly downregulated in high-grade tumours. This is mimicked by the GFAPα- condition in the astrocytoma cell line experiment. However, the picture that is emerging from comparing the TCGA and the GFAPα- condition showed that some of the targets identified in the in vitro study were not correlated to GFAP in the patient data from the TCGA. As the TCGA data is obtained from RNA-samples taken from a biopsy, this gives very local information of the tumour, whereas the full astrocytoma tumour environment is hallmarked by its pluriformity. Therefore, the expression of genes at the rim of tumour could differ from the core of the tumour. A good analogy of this concept is given by the specific keratin-14 expression of the invasive front of a 3d breast cancer model, which is essential to give the front a basal-epithelial phenotype (Cheung et al., 2013), or the increased expression of Vim at the leading edge in a lens epithelium wound healing (Menko et al., 2014). Hence, our in vitro targets are of interest for tumour biology in patients, but the heterogeneity of specific tumour cell populations needs to be taken into account.

GFAP modulation induces gene clusters involved in Extracellular matrix remodelling and cell-cell signalling

In Chapter 5 we isolated astrocytes from mice lacking GFAP. By comparing the gene ontology analysis of Chapter 4 on human astrocytoma cell lines with Chapter 5 on primary mouse astrocytes we observed an overrepresentation of two ontologies relevant for both tumour biology and astrocytes in physiology and pathology. The first is extracellular matrix (ECM) remodelling. This process is essential for brain tumours to grow and metastasize. On the one hand tumours have to degrade the ECM barrier to allow for infiltration. On the other hand the ECM environment of the tumour itself has to meet requirements for maintenance of the various heterogeneous populations of tumour cells present in the tumour, ranging from a tumour stem cell population to the vasculature of the tumour (Wang et al., 2010). A functional analysis in Ingenuity Pathway Analysis (IPA) performed in Chapter 4 predicted an enhancing effect on invasion of tumours in the GFAPδ+ condition, in contrast to a decreased predicted invasion in the GFAPα condition. The predicted lower invasive capacity of GFAPα knockdown cells is in agreement with the effects
<table>
<thead>
<tr>
<th>Gene Symbol human (mouse)</th>
<th>Gene function</th>
<th>$FC_{\alpha+}$</th>
<th>$FC_{\delta+}$</th>
<th>$FC_{\text{pan-}}$</th>
<th>$FC_{\alpha-}$</th>
<th>$FC_{\text{Gko}}$</th>
<th>$FC_{\text{GVko}}$</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP2</td>
<td>Peptidase</td>
<td>n.s.</td>
<td>0.6</td>
<td>n.s.</td>
<td>1.5</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Role in tumour invasiveness, immune recruitment and vasculogenesis (Tremblay et al., 2011)</td>
</tr>
<tr>
<td>CPE</td>
<td>Peptidase</td>
<td>n.s.</td>
<td>0.6</td>
<td>(2.1)</td>
<td>4.9</td>
<td>n.s.</td>
<td>n.s.</td>
<td>Can control switch between migration and proliferation (Höring et al., 2012)</td>
</tr>
<tr>
<td>A2M</td>
<td>Peptidase</td>
<td>2.7</td>
<td>3.0</td>
<td>n.s.</td>
<td>0.2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Inhibits MMP2 (Fears et al., 2005)</td>
</tr>
<tr>
<td>PI15</td>
<td>Peptidase</td>
<td>2.3</td>
<td>2.5</td>
<td>(0.5)</td>
<td>0.4</td>
<td>n.d.</td>
<td>n.d.</td>
<td>CAP superfamily (Gibbs et al., 2008)</td>
</tr>
<tr>
<td>GLIPR1</td>
<td>Peptidase</td>
<td>1.6</td>
<td>1.4</td>
<td>n.s.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>CAP superfamily (Gibbs et al., 2008)</td>
</tr>
<tr>
<td>GLIPR2</td>
<td>Peptidase</td>
<td>n.s.</td>
<td>n.s.</td>
<td>0.53</td>
<td>0.69</td>
<td>n.s.</td>
<td>n.s.</td>
<td>CAP superfamily (Gibbs et al., 2008)</td>
</tr>
<tr>
<td>FBLN1</td>
<td>ECM associated</td>
<td>n.s.</td>
<td>1.43</td>
<td>n.s.</td>
<td>0.54</td>
<td>1.98</td>
<td>n.s.</td>
<td>Incorporates into ECM, alters higher order structure and can activate peptidases (Twal et al., 2001)</td>
</tr>
<tr>
<td>LAMA1</td>
<td>ECM</td>
<td>0.34</td>
<td>0.41</td>
<td>n.s.</td>
<td>9.16</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Component of ECM, highly important for angiogenesis, migration, neurogenesis and invasion (Kawataki et al., 2007)</td>
</tr>
<tr>
<td>LAMA4</td>
<td>ECM</td>
<td>n.s.</td>
<td>1.48</td>
<td>n.s.</td>
<td>0.67</td>
<td>n.s.</td>
<td>1.7</td>
<td>Component of ECM, highly important for angiogenesis, migration, neurogenesis and invasion (Kawataki et al., 2007)</td>
</tr>
<tr>
<td>SPP1</td>
<td>ECM</td>
<td>n.s.</td>
<td>2.17</td>
<td>n.s.</td>
<td>1.57</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Osteopontin, strongly associated to migration, invasion and stemness (Pietras et al., 2014)</td>
</tr>
<tr>
<td>ACP5</td>
<td>ECM Modifier</td>
<td>n.s.</td>
<td>0.56</td>
<td>n.s.</td>
<td>0.51</td>
<td>0.61</td>
<td>n.s.</td>
<td>Unique phosphatase of SPP1 (Lausch et al., 2011)</td>
</tr>
<tr>
<td>LEPREL1</td>
<td>ECM Modifier</td>
<td>n.s.</td>
<td>1.59</td>
<td>n.s.</td>
<td>0.37</td>
<td>n.s.</td>
<td>n.s.</td>
<td>Collagen IV Prolyl-3-hydroxylation (Hudson et al., 2015)</td>
</tr>
<tr>
<td>SULF1</td>
<td>ECM / GF Modifier</td>
<td>1.90</td>
<td>1.95</td>
<td>n.s.</td>
<td>0.17</td>
<td>1.58</td>
<td>1.53</td>
<td>HSPG sulphate cleavage, GF retention altered (Joy et al., 2015; Ramsbottom et al., 2014)</td>
</tr>
<tr>
<td>SULF2</td>
<td>ECM / GF Modifier</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>1.86</td>
<td>n.s.</td>
<td></td>
<td>HSPG sulphate cleavage, GF retention altered (Kleinschmit et al., 2010; Meyers et al., 2013)</td>
</tr>
</tbody>
</table>
## Summary and Discussion

### ERBB2 GF Signalling
- **Signalling**: n.s., 0.74, n.s., 0.31, n.s., 0.80
- **ERBB2 GF Signalling**: EGF & Neuregulin coreceptor, risk factor in astrocytoma (Gulati et al., 2010)

### KDR GF Signalling
- **Signalling**: n.s., 1.55, n.s., 1.87, n.s., 1.63
- **KDR GF Signalling**: VEGF receptor, crucial for vascularization, possible role in vasculogenic mimicry (Wang et al., 2010; Francescone et al., 2012)

### IGFBP5 GF Signalling
- **Signalling**: n.s., 1.51, n.s., 1.57, n.s., 2.14
- **IGFBP5 GF Signalling**: Binds to Insulin-like Growth Factor (IGF), stabilizing it and modulating receptor interactions (Schneider et al., 2002)

### BAIAP2L1 GF Signalling
- **Signalling**: n.s., 1.54, n.s., 1.62, n.s., n.s.
- **BAIAP2L1 GF Signalling**: Target and Scaffold for Insulin Signalling Cascade (Millard et al., 2007; Wang et al., 2013)

### PRKCZ Signalling
- **Signalling**: n.s., 0.63, n.s., 0.54, n.s., 0.58
- **PRKCZ Signalling**: Protein Kinase Downstream of EGFR signalling, inhibition reduces invasion of astrocytoma (Guo et al., 2009)

### GABBR2 Signalling
- **Signalling**: n.s., 1.46, n.s., 0.56, 0.61, 0.48
- **GABBR2 Signalling**: Member of a GABA-receptor complex, relevant for astrocyte-neuron interactions

### OGDHL Signalling / metabolism
- **Signalling / metabolism**: 0.68, 0.37, n.s., 2.27, n.s., n.s.
- **OGDHL Signalling / metabolism**: Dehydrogenase of oxoglutarate, metabolite relevant as substrate of oncogenes IDH1/IDH2 (Dang et al., 2009)

### THSD7A Signalling
- **Signalling**: 1.36, n.s., n.s., 0.50, 1.59, 1.53
- **THSD7A Signalling**: Important for angiogenesis (Liu et al., 2016)

### C16orf45 (2900011O08Rik) Signalling?
- **Signalling?**: n.s., 0.74, n.s., 0.60 (0.70), n.s., 0.56
- **C16orf45 (2900011O08Rik) Signalling?**: Recently identified as Migration Inhibitory Protein (MINP), involved in radial migration (Zhang et al., 2014)

### NTNG1 Axonal Guidance
- **Axonal Guidance**: 0.69, 0.59, n.s., 1.38, 0.35, 0.49
- **NTNG1 Axonal Guidance**: Growth cue for axons, promotes neurite outgrowth (Moore et al., 2012)

### SLC14A1 Transport
- **Transport**: 0.41, n.s., n.s., 4.11, 1.64, n.s.
- **SLC14A1 Transport**: Ureum transporter, unknown function in astrocytes/astrocytoma

### CRYAB Small Heat Shock
- **Small Heat Shock**: n.s., 1.79, n.s., 0.23, n.s., n.s.
- **CRYAB Small Heat Shock**: IF associated protein, strongest regulated gene in IDH1 mutated astrocytoma patients (Avliyakulov et al., 2014)

Entries in italics were identified by the meta-analysis. Entries in bold are the most promising targets based on consistent correlation with GFAP.

FC: Fold Change; α+: U251 GFAPα recombinant expression; δ+: U251 GFAPδ recombinant expression; pan-: U373 GFAPpan knockdown; α-: U373 GFAPα knockdown; Gko: GFAP-/- isolated astrocytes; GVko: GFAP-/-Vim-/- isolated astrocytes; Fold changes between parentheses: not significant but trend; n.d.: not detected; n.s.: not significant; ECM: Extracellular Matrix; GF: Growth Factor; CAP: CRISPS, Ag5, and Pr-1; HSPG: heparan sulphate proteoglycan; IDH1: isocitrate dehydrogenase 1, oncogene for astrocytoma
observed in *in vitro* studies (Moeton et al., 2014). In contrast, we observed that higher grade and more invasive astrocytoma cells lose their GFAP expression. Thus, more experiments are required on strictly defined GFAP isoform modulations to gain more insight in the specific role of GFAP versus the GFAPδ/GFAPα ratio. This regulation might even be more complex if we take into account the other 8 identified GFAP isoforms (Hol and Pekny, 2015; Middeldorp and Hol, 2011). ECM remodelling is also relevant for astrocytes both in physiology and pathology as the ECM is an essential component in physiological neuronal communication (Dityatev et al., 2010), whereas in pathology ECM remodelling is part of the gliotic response (Sofroniew, 2009). The DEGs in our experiments encompass ECM proteins, ECM protein modifying enzymes, peptidases, and peptidase inhibitors and thus modulating the GFAP network can have a large effect on the composition of the ECM (Table 2).

The second ontology overrepresented in Chapter 4 and Chapter 5 was related to the cell’s interaction with its environment, and cell signalling with a focus on growth factors (Table 2). It is tempting to interpret this combination of control over ECM composition and signalling pathways as specifically relevant for multicellular organisms, the group of organisms that cytoskeletal IFs are restricted to (Kollmar, 2015).

**GFAP is involved in the Glutamine-Glutamate/GABA cycle**

We combined our all our datasets (astrocytoma with an overexpression or knockdown of GFAP and astrocytes isolated from GFAP knockouts) in a meta-analysis (see Box 1). The most consistent genes responding to GFAP were SLC14A1 (negatively correlating with GFAP expression) and GABBR2 (positively correlating with GFAP expression) (Table 2). These targets can either be directly or indirectly regulated by GFAP or can have functional interactions with GFAP. Therefore, SLC14A1 and GABBR2 are the most promising targets for future studies.

SLC14A1 (Solute Carrier 14A, member 1 (Kidd Blood Group)) is a transporter responsible for urea transport, mainly described to be important in kidney and erythrocytes. Urea is the less toxic form of ammonia, and a product of amino acid metabolism. Although urea is mainly considered to be produced in the liver (Weiner et al., 2015), the RNAseq database ([www.brainrnaseq.org](http://www.brainrnaseq.org)) of Barres et al. and our microarray data confirm that SLC14A1 is expressed in mouse astrocytes (Cahoy et al., 2008; Orre et al., 2014a). Note that hepatic stellate cells in the liver also express GFAP (Schachtrup et al., 2011), and that GFAP may also play a role in liver amino acid metabolism. In astrocytes metabolism of Glutamine, Glutamate and γ-aminobutyric acid (GABA) takes place, nitrogen rich amino acids. These
three amino acids play an important role in neuronal signalling. Glutamate is an excitatory neurotransmitter and GABA an inhibitory neurotransmitter. Both can be

Box 1: Meta-analysis

To determine highly conserved molecular pathways that are regulated by the change in the GFAP network, we investigated the overlap between the datasets in Chapter 4 (human astrocytoma cell lines) and 5 (acutely isolated mouse astrocytes) to identify common denominators. This often used strategy of meta-analysis can lead to novel findings of inconspicuous targets, as the persistent effects between different model systems may not be the same as the top of the single studies. We compared the two transcriptomics studies in this thesis using Fisher’s inverse chi-square, where p-values from separate studies can be combined to calculate a combined p-value (Campain and Yang, 2010; Fisher, 1970). We applied this method to our datasets, using as input every separate p-value for GFAPδ⁺, GFAPα⁺, GFAPpan⁻, GFAPα⁻, GFAPko mouse, and VIM-GFAPko mouse, to come up with the most consistently responding genes to GFAP modulation. First, human and mouse orthologs based on gene names were identified using Ensembl Biomart, resulting in 9,957 genes represented in all datasets. If one human gene resulted in multiple mouse orthologs or if genes were represented on the array multiple times, the lowest p-values of the identified set of similar genes were used for the comparison, not taking into account direction of change. Since Fisher’s inverse chi-square is known to be anti-conservative, even more so when samples are not independent (since the U251 recombinant expression, the U373 knockdown and the mouse knockout study all have multiple conditions used as separate inputs in the test), we filtered the genes of interest further for significant change in each of the three GFAP modulations. This resulted in 47 genes that were significantly changed in all paradigms. We excluded genes that were not significantly regulated in the GFAPko to leave out genes regulated by Vim or complete IF-network ablation. By comparing the genes identified in the meta-analysis with the genes identified on basis of functions in ECM or signalling, 7 genes emerged that were not identified before (Table 2, entries in italics). Note that the GFAPpan⁻ condition is not significantly regulated in most of the genes in Table 2, possibly due to the weak response of GFAP to this knockdown condition. As the direction of change is not taken into account in this p-value based analysis, this was investigated manually, leading to the identification of GABBR2 and SLC14A1 as most promising GFAP responders.
taken up by astrocytes and metabolised to Glutamine, which is then transported back to neurons that can recycle it, a process known as the Glutamine-Glutamate/GABA cycle (Walls et al., 2015). Whether urea or the urea cycle is involved in that has not been described.

GABBR2 (GABA B receptor 2) forms a G-protein coupled receptor complex with GABBR1 (Geng et al., 2013). The GABBR1 gene was also detected in astrocytes in an earlier study by our group (Orre et al., 2014b). Furthermore, GABBR2 in astrocytes binds to the astrocyte specific enzyme converting Glutamate into Glutamine, Glutamine synthethase (GS), thereby affecting localization and stability of GS (Huyghe et al., 2014). A study investigating the role of reactive astrocytes in neuronal signalling found that reactive astrocytes produced less GS. This resulted in reduced GABAergic signalling and subsequent reduced inhibition of neurons, leading to overactivity in neuronal networks, which could be rescued by providing external glutamine (Ortinski et al., 2010). Earlier studies have shown that GFAP positive reactive astrocytes responding to intrastratal lesions are highly GABBR2 immunoreactive (Rekik et al., 2011), thereby corroborating our finding that GFAP and GABBR2 are co-regulated. In addition, GFAP with the R239C mutation, the most common mutation in Alexander’s Disease, causes aggregation of GFAP. In vitro and in vivo this results in a reduction of Glutamate Transporter 1 (GLT-1) protein levels and attenuated inward glutamate transport (Tian et al., 2010). An earlier study also revealed that GFAPko mouse have altered localization of GLT-1 (Hughes et al., 2004) and that another Glutamate transporter (GLAST) interacts with GFAP (Sullivan et al., 2007), further corroborating the role of GFAP in astrocyte Glutamine-Glutamate-GABA metabolism. Concluding, we and others showed that a change in GFAP leads to changes in proteins involved in the regulation of the Glutamine-Glutamate/GABA cycle, thereby affecting neuronal communication.

**Mechanisms of GFAP regulated gene expression**

One question not addressed elsewhere in this thesis is how GFAP regulates the expression of all the other genes that are differentially expressed in our analyses. Therefore we performed an Ingenuity Pathway Analysis (IPA) for regulators upstream of all the DEGs identified in our studies.

The astrocytoma IPA upstream analysis revealed two candidate key regulators: TGFβ and IL6 (interleukin 6). Both signalling pathways were predicted to be significantly activated in the GFAPδ+ cells, and predicted to be significantly inhibited in the GFAPα cells. TGFβ has both antitumour as well as tumorigenic properties. It is involved in stemness, angiogenesis, invasion, treatment resistance
and immunosuppression as reviewed by Joseph et al (Joseph et al., 2013). Various genes involved in the extensive TGFβ signalling pathway are altered in our GFAPα cells, with a significant upregulation of NOG (Noggin; 1642%, FDR<.001) and downregulation of BAMBI (BMP and activin membrane-bound inhibitor; 42%, FDR<.001). NOG can bind and inactivate members of the TGFβ family, and BAMBI is a pseudoreceptor for TGF family members, without an intracellular signalling domain. IL6 promotes invasion and angiogenesis in astrocytoma, whereas IL6 or IL6 receptor targeted interventions reduces astrocytoma stem cell survival and tumour size in xenograft studies (Liu et al., 2010; Wang et al., 2009).

An important and interesting question is how GFAP modulation could alter signalling by TGFβ and/or IL-6. GFAPδ can bind to presenilin-1, which is the active enzymatic part of the γ-secretase complex (Li et al., 2006; Nielsen et al., 2002). The γ-secretase enzyme can cleave two TGFβ receptors: TβRI and betaglycan (Blair et al., 2011; Gudey et al., 2014), as well as process the IL-6 receptor (Chalaris et al., 2010). We hypothesize that turnover of the respective TGFβ and IL-6 receptors can be regulated or coordinated by the presence of GFAP, either through GFAP modulation of the γ-secretase activity, or by GFAP altering receptor endocytosis. From studies on the VIM-GFAPko, it is known that astrocyte IFs play a role in vesicle trafficking, and that Notch signalling is altered due to a reduced Jagged1 vesicle content and reduced Notch endocytosis (Potokar et al., 2010; Vardjan et al., 2012; Wilhelmsson et al., 2012). It is known that Notch signalling strongly interacts with TGFβ signalling pathways, providing an alternative path of GFAP mediated changes downstream of TGFβ (Andersson et al., 2011; Borggrefe et al., 2016).

The genes altered by in vivo GFAPko were also analysed by IPA upstream analysis, but here the lowest p-value of upstream regulators was determined to be TNFα, with lower confidence scores. This may represent a difference between tumour versus astrocyte biology, or may be due to the much more complex biology of long-term GFAPko in astrocytes in contrast to the relatively straightforward and direct GFAP modulation in cell lines.

**Conclusion**

We have found that changes in GFAP have an impact on ECM remodelling and cell-cell signalling genes. Our data provide insight into (1) the mechanisms regulated by GFAP and its isoforms, and (2) the effect of the stoichiometry of the GFAP isoforms on the integration of signalling in astrocytoma. Combining this data with the in vivo data revealed further evidence for the effects changes in GFAP isoform and/or Vim expression have on cell signalling and the ECM composition.
Unexpected candidates emerging from the combination of these datasets were the GABBR2 and SLC14A1 genes, further opening the possibility of a role of GFAP in the regulation of the Glutamine-Glutamate/GABA cycle.