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Scientific correspondence

The putative oncogene CPI-17 is up-regulated in schwannoma

CPI-17 (PPP1R14A protein phosphatase 1 regulatory inhibitor subunit 14A, 17 kDa) was previously described to act as an oncoprotein in the merlin pathway. The myosin phosphatase and its substrate merlin are part of a tumour suppressor cascade that can be blocked in two ways; by mutation of the *NF2* gene encoding the tumour suppressor protein merlin and by up-regulation of the specific myosin phosphatase inhibitor, CPI-17. Inactivation of the merlin tumour suppressor pathway is thought to be a major principle of transformation by CPI-17 [1]. While CPI-17 misexpression was demonstrated in several human tumour cell lines, Thurneysen *et al.* [2] showed for the first time that CPI-17 plays a role as oncogene in human malignancies. The authors found that CPI-17 expression in mesothelioma is significantly increased in tumours without deleted *NF2* gene, compared to normal pleura and tumours with truncating *NF2* mutations.

Interestingly schwannomas that typically show alterations in the merlin pathway and other peripheral nerve sheath tumours (PNST) have never been analysed for CPI-17 expression. Considering the potential importance of merlin inactivation as determinant of transformation by CPI-17, we screened 160 paraffin-embedded samples of PNST [perineurioma, ganglioneuroma, schwannoma of different aetiology, neurofibromatosis type 1 (NF1)-associated neurofibroma and malignant peripheral nerve sheath tumour (MPNST)] along with 28 peripheral nervous system biopsies harbouring a nontumour pathology for expression of CPI-17 (Table 1, [3–5]). All procedures involving human participants in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Permission to perform this study was granted by §12 of the Hamburgisches Krankenhausgesetz, Hamburg, Germany.

Firstly, we performed specificity validation of two commercially available CPI-17 antibodies: (i) a murine monoclonal (C1) and (ii) a goat polyclonal (A20) CPI-17 antibody; both raised against an N-terminal CPI-17 epitope of human origin. By Western blot analysis, we found that a human melanoma cell line (RPM-MC) [6], previously shown to express CPI-17, gave a positive signal (Figure 1**a**). In contrast, the human arachnoidal cell line (AC-1) [7] was found to be CPI-17 negative. However, transfection of AC-1 cells with a plasmid encoding for FLAG-tagged-CPI-17 demonstrated antibody specificity. In conclusion, the CPI-17 antibodies C1 and A20 detected two distinct proteins at 17 kDa, which correspond to the dephosphorylated and phosphorylated form of CPI-17 [8] (Figure 1**a**). Because the A20 antibody showed additional unspecific protein detection at approximately 27 kDa (not shown), we applied the monoclonal C1 antibody for all subsequent histopathological analysis.

To evaluate the C1 antibody for immunohistochemistry, we stained adult mouse kidneys for CPI-17 and found specific staining in vascular smooth muscle cells, as described earlier [8] (Figure 1**b**). Importantly, the C1 antibody signal on histological sections of CPI-17 knock-out mice was, as expected, absent in the kidney (Figure 1**b**).

In human histopathological sections, blood vessels were always CPI-17 positive and served as internal positive control for all cases (Figure 1**c**, sural nerve biopsy). In 28 nontumour samples of the peripheral nervous system, no CPI-17 expression other than in vascular smooth muscle was observed (Figure 1**c**). In particular, there was no up-regulation in inflammatory pathologies like chronic demyelinating inflammatory neuropathy (CIDP) ($n = 3$), vasculitis ($n = 3$), or traumatic neuromas ($n = 15$), compared to normal sural biopsies.

NF1-associated peripheral nerve sheath tumours, intraneural nodular plexiform neurofibroma and dermal neurofibroma stained negative for CPI-17. Further, NF1-associated MPNST did not express CPI-17 (Figure 1**c**), except for two tumours, which also expressed smooth muscle actin (malignant Triton tumour) (not shown). In two cases of hybrid-tumours (synonym schwannoma in neurofibroma) [9], neither the areas showing differentiation of neurofibroma, nor

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Table 1. Patient age and gender

Disorder	*Female:Male*	*Mean age (years)*	*Age range*
NF2-associated schwannoma	6:4	31.9	4–54
Schwannomatosis- associated schwannoma	3:0	36.0	18–53
Sporadic schwannoma	14:16	53.5	15–87
NF1-associated plexiform neurofibroma	23:26	27.6	3–68
NF1-associated cutaneous neurofibroma	28:19	38.8	15–64
NF1-associated MPNST	9:8	39.3	17–63
Hybrid tumour	1:1	32.5	26–39
Perineurioma	1:0	51.0	51
Ganglioneuroma	1:0	27.9	27

Number, female-to-male ratio, mean age of patients, and age range included in this study is shown. All neurofibromatosis 1 (NF1)- and neurofibromatosis 2 (NF2)-patients were diagnosed according to NIH- [3] or Manchester criteria [4], respectively. Schwannomatosis was diagnosed according to MacCollin *et al.* [5].

the ones that presented with schwannoma morphology, expressed CPI-17 (Figure 1**c**).

In contrast to the aforementioned tissues, the vast majority of schwannomas (39/43) expressed CPI-17 (Figure 1**c**). The labelling frequently appeared patchy and varied in intensity; especially in NF2-associated tumours, which showed a significantly lower median labelling intensity than sporadic schwannomas (Figure 1**d**; $P < 0.02$ Mann–Whitney-test). Schwannomatosis-associated tumours demonstrated expression levels between sporadic and NF2-associated schwannomas (Figure 1**c**,**d**). Interestingly, spinal ganglion cells, like neuronal elements in a ganglioneuroma, did not express CPI-17 (Figure 1**c**).

CPI-17 was predominantly found in the nucleus, which is in line with previous reports [10]. We therefore hypothesized that its expression correlates with cellular proliferation. In order to test the proliferative effect of CPI-17 expression in schwannoma, the proliferation index was assessed in all schwannomas by labelling with Ki-67 antibodies (Figure 1**f**). High CPI-17 levels (staining intensity > 2) in schwannomas were found to correlate with higher Ki-67 proliferation indices (Figure 1**e**).

Apart from a number of studies addressing CPI-17 function in vascular smooth muscle cells in pig and mice [5,11], the physiological expression pattern of CPI-17 in other tissues has not been investigated systematically. Here, we show that CPI-17 is absent in resident, normal human peripheral nervous tissue as well as in nontumour pathologies of the PNS. However, we now provide evidence that the putative oncogene CPI-17 is specifically up-regulated in the tumour entity 'schwannoma'.

Figure 1. (**a**) Western Blot analysis for actin as loading control (clone I-19, 1:1500, Santa Cruz Biotechnology) and CPI-17 using monoclonal CPI-17 antibody (clone C1, 1:500, Santa Cruz Biotechnology) on human cell lysates. No detection of CPI-17 in protein lysates from human arachnoidal cells (AC-1, negative control), but in positive control lysates (AC-1 cells transfected with FLAG-tagged CPI-17 and RPM-MC cells which express endogenous CPI-17). (**b**) CPI-17 antibody staining on paraffin-embedded kidney slices from wild-type (left side) or CPI-17 knock-out mice (right side); smooth muscle cells in blood vessels are intensively stained with CPI-17 (C1) antibody in wild-type (black arrow), but not in CPI-17 knock-out tissue; scale bar 200 μm. (**c**) CPI-17 is expressed in schwannomas, but not in other peripheral nervous system tumours or nontumour pathology. All specimens were primarily diagnosed by two neuropathologists of the Institute of Neuropathology according to current WHO criteria. The biopsies comprised 30 sporadic schwannomas, three schwannomatosis-associated peripheral schwannomas, 10 NF2-associated peripheral schwannomas, two hybrid-tumours, one perineurioma, one ganglioneuroma, 49 NF1-associated intraneural plexiform and 47 dermal neurofibromas, 17 NF1-associated malignant peripheral nerve sheath tumours (MPNST), 13 sural nerve biopsies with nontumour pathology (three CIDP, seven noninflammatory axonopathies, three cases of vasculitis) and 15 traumatic neuromas. Note physiological expression of CPI-17 in smooth muscle cells of vessels (arrows). Immunohistochemistry was performed in an automated stainer (Ventana Medical Systems, Tucson, AZ, USA) using a standard antigen retrieval protocol (CPI-17 antibody clone C1, 1:100, pretreatment protocol cc1st). Bound antibodies were detected by the peroxidase method using diaminobenzidine as chromogen (Ventana Medical Systems, # 760-500). All slides were counterstained with alum-haematoxylin; scale bar 20 μm. (**d**) Mean expression levels as scored from antibody labelling intensity of CPI-17 in sporadic schwannomas (Sporadic schwannomas, $n = 30$), Schwannomatosis ($n = 3$), NF2-associated schwannomas ($n = 10$), cutaneous neurofibroma (CNF, $n = 47$), plexiform neurofibroma (PNF, $n = 49$) and MPNST ($n = 17$). CPI-17 expression was evaluated semi-quantitatively using four grades for all tumours (0: no staining, 1: slight to medium labelling of 5–50% of tumour cells, 2: slight to medium labelling of ≥50% of tumour cells, 3: strong labelling of ≥50% of tumour cells). (**e**) High expression levels of CPI-17 correlate with increased Ki-67 proliferative activity in sporadic schwannomas. Samples with no to medium CPI-17 expression (score ≤ 2, black bar) show significantly lower Ki-67 proliferative activity than samples with high CPI-17 expression (score = 3, grey bar). Ki-67 proliferation indices were calculated as percentage of labelled nuclei to all nuclei in 0.1 mm^2 of the tumour area with the highest density of Ki-67 labelled cells (error bars indicate +/− one standard error of the mean). (**f**) Examples of two sporadic schwannomas with medium CPI-17 expression and low Ki-67 proliferation index (1.3%, case #122-14) *vs.* high CPI-17 expression and increased Ki-67 proliferation index (9.27%, case #357-11); scale bar 20 μm.

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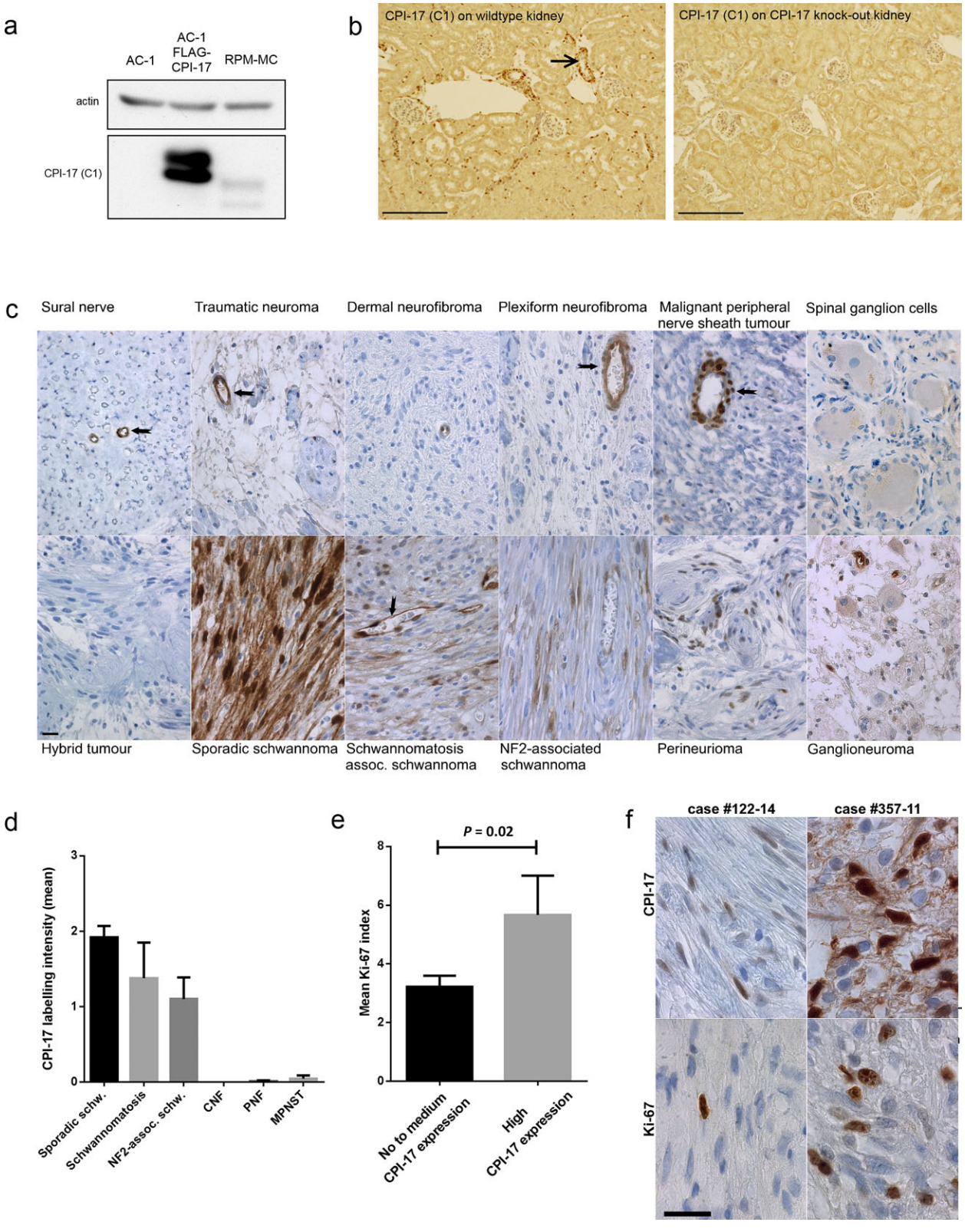

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Previous investigations on schwannomas showed that 79% of NF2-associated [12], but only 45% [13] to 56% [12] of sporadic schwannomas harbour two mutational hits affecting the *NF2* gene. Further, no association was found with the type of mutation and relevant clinical parameters in sporadic tumours [13]. These data strongly suggest the involvement of additional oncogenic pathways, primarily in sporadic schwannomas, but also in NF2-associated tumours. In accordance with this assumption, we found medium or high expression levels of CPI-17 in 73% (22/30) of sporadic schwannomas – but in only 20% (2/10) of NF2-associated schwannomas ($P < 0.02$). However, expression of CPI-17 was evident in over 90% of all schwannomas, suggesting a role in schwannoma growth in addition to, or in parallel with, *NF2* mutations. In support of this conclusion, high CPI-17 levels in schwannoma correlated with increased Ki-67 proliferation indices. CPI-17 was frequently localized not only in the cytoplasm but also, or even exclusively, in the nucleus of tumour cells. Previously it was suggested that CPI-17 is actively imported to the nucleus where it inhibits dephosphorylation of histone H3, resulting in increased cell proliferation [10]. Our results indicate a potential role of CPI-17 in schwannoma progression. However, the data did not suffice to prove a causative function of CPI-17 in schwannoma pathogenesis in the absence of *NF2* mutations. The latter question could be addressed by correlation of the *NF2* mutational status with the level of CPI-17 expression.

From a diagnostic perspective, CPI-17 overexpression in schwannoma is a valuable finding, since the three main PNST entities – neurofibroma, schwannoma and MPNST – show similar antigen expression patterns with respect to S100 protein, collagen IV, CD34 and Sox10 [14], and may also display overlapping growth patterns. These similarities may pose problems in the correct diagnosis of PNST [14], which in turn is an essential prerequisite for an adequate therapy. Although the treatment of choice for symptomatic benign and malignant PNST is surgical excision, medical treatment of schwannomas with bevacizumab has been shown to procure tumour regression and improve hearing in NF2-patients with vestibular schwannoma not eligible for surgery [15].

In conclusion, CPI-17 is not expressed in the healthy peripheral nervous system or in nontumour pathologies of the PNS. CPI-17 up-regulation, however, is observed in over 90% of schwannomas, but not in neurofibroma and only rarely in MPNST. CPI-17 expression correlates with tumour cell proliferation and because of its predominant expression in schwannoma, CPI-17 is proposed as a promising diagnostic marker differentiating between schwannoma and other PNST or nontumour peripheral nerve lesions.

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Conflict of interest

The authors declare that they have no conflict of interest.

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