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# Smad-interacting protein 1 affects acute and tonic, but not chronic pain

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**Conflicts of interest**
None declared.

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## Abstract

**Background:** Smad-interacting protein 1 (also named *Zeb2* and *Zfhx1b*) is a transcription factor that plays an important role in neuronal development and, when mutated, causes Mowat–Wilson syndrome (MWS). A corresponding mouse model carrying a heterozygous *Zeb2* deletion was comprehensively analysed in the German Mouse Clinic. The most prominent phenotype was the reduced pain sensitivity. In this study, we investigated the role of *Zeb2* in inflammatory and neuropathic pain.
**Methods:** For this, we tested mutant *Zeb2* animals in different models of inflammatory pain like abdominal constriction, formalin and carrageenan test. Furthermore, we studied the pain reactivity of the mice after peripheral nerve ligation. To examine the nociceptive transmission of primary sensory dorsal root ganglia (DRG) neurons, we determined the neuronal activity in the spinal dorsal horn after the formalin test using staining of c-Fos. Next, we characterized the neuronal cell population in the DRGs and in the sciatic nerve to study the effect of the *Zeb2* mutation on peripheral nerve morphology.
**Results:** The present data show that *Zeb2* is involved in the development of primary sensory DRG neurons, especially of C- and Aδ fibres. These alterations contribute to a hypoalgesic phenotype in inflammatory but not in neuropathic pain in these $Zeb2^{+/-}$ mice.
**Conclusion:** Our data suggest that the under-reaction to pain observed in MWS patients results from a reduced responsivity to nociceptive stimulation rather than an inability to communicate discomfort.

**What's already known about this topic?**
- *Zeb2* is a transcription factor that is involved in neuronal development and, when mutated, causes Mowat–Wilson syndrome (MWS).
- *Zeb2* controls the transduction properties of heat-sensitive primary sensory neurons.

**What does this study add?**
- This study shows that *Zeb2* is involved in the development of DRG neurons, especially of C- and Aδ fibres.
- Further, we demonstrate that *Zeb2* modulates formalin and proton-evoked inflammatory pain but not the development of chronic pain.
- Our data suggest that the under-reaction to pain observed in MWS patients results from a reduced responsivity to nociceptive stimulation rather than an inability to communicate discomfort.

## 1. Introduction

Smad-interacting protein-1 (Sip1) is a two-handed zinc finger, multi-domain transcription factor. Mutations in *Zeb2* (the human gene often being referred to as *ZFHX1B*) have been implicated in the aetiology of Mowat–Wilson syndrome (MWS) (Mowat et al., 1998; Cacheux et al., 2001; Wakamatsu et al., 2001), a developmental disorder characterized by distinct facial dysmorphology and moderate-to-severe intellectual disability (Garavelli and Mainardi, 2007). The behavioural phenotype associated with MWS ranges from oral and repetitive behaviours to under-reaction to pain (Evans et al., 2012). Abnormal reaction to pain has been commonly reported in patients suffering cognitive disorders. Under-reaction to pain in persons with intellectual disability could reflect on two aspects: an inability to perceive pain or an inability to recognize and communicate their pain experience (Breau et al., 2001, Nagasako et al., 2003). Therefore, studying the pain reaction of mice having a conceptually similar mutation as most MWS patients (Garavelli et al., 2009) can broaden our knowledge in the characterization of MWS and the role of *Zeb2* in neurodevelopment and pain management.

Pain is important sensory information that is mediated by specialized primary sensory neurons, nociceptors (Bessou and Perl, 1969), whose cell bodies are situated in the dorsal root ganglia (DRG). These neurons are characterized by pseudo-unipolar axons, which detect painful stimuli at their peripheral endings and project to the dorsal horn of the spinal cord (Costigan et al., 2009). Primary sensory neurons contain various ion channels or receptors at their peripheral nerve endings that can detect heat, cold, mechanical and chemical stimuli (Scholz and Woolf, 2002; Hwang and Oh, 2007; Basbaum et al., 2009).

Homozygous deletion of *Zeb2* in mice is embryonic lethal (Van de Putte et al., 2003), and various conditional-knockout mouse models have revealed crucial developmental functions including in the forebrain and in neural crest-derived tissues (Miquelajauregui et al., 2007; Van de Putte et al., 2007; Seuntjens et al., 2009). Mice with a heterozygous deletion of this gene are viable and do not exhibit obvious malformations. Subtle phenotypes in these mice may reveal novel functions of *Zeb2*, and therefore, we performed a comprehensive phenotype screen in the German Mouse Clinic (GMC), which is a multidisciplinary phenotyping centre for mouse mutants (Gailus-Durner et al., 2005; Fuchs et al., 2009). Generally, the mutant mice did not show any changes in their behaviour compared with wild-type littermates. The most prominent phenotype of the *Zeb2* heterozygote mice was a reduced pain sensation. Recently, we reported that mice with a heterozygous mutation in the *Zeb2* gene showed a reduced sensitivity in acute thermal pain tests, while pain reactions to mechanical stimuli were not altered (Jeub et al., 2011). The transduction properties of heat-sensitive primary sensory neurons were altered in *Zeb2* mutant mice compared with wild-type controls (Jeub et al., 2011).

In this study, we investigated if the same *Zeb2* mutation also influenced inflammatory pain sensation using different inflammatory pain models. Neuronal activation in the spinal cord after formalin injection was revealed by c-Fos immunoreactivity. Further, we examined the development of neuropathic pain after partial ligation of the sciatic nerve.

## 2. Materials and methods

### 2.1 Animals

Male and female mice (3–5 months old) carrying one mutant allele of *Sip1*/*Zeb2* (*Zeb2*$^{+/-}$) and wild-type littermates on a CD1 background (backcrossed for >8 generations) were used as previously published (Van de Putte et al., 2003). Animals were kept in a reversed light/dark cycle (light off between 7:00 a.m. and 7:00 p.m.) and received food and water *ad libitum*. The housing conditions were maintained at 21 ± 1°C and 55 ± 10% relative humidity. Experimental procedures complied with all regulations for animal experimentation in Germany and were approved by Landesamt für Natur,

Umwelt und Verbraucherschutz in Nordrhein-Westfalen, Germany (AZ: 50.203.2-BN34 44/04).

## 2.2 Writhing test

For this assay, mice were placed in a small observation chamber (10 × 10 cm) and habituated for at least 30 min. Acetic acid (0.6%) was injected into the peritoneum in a volume of 10 ml/kg. Within minutes after the injection, a typical 'writhing' response, indicative of visceral pain, could be observed. Writhing is a distinct severe contraction of the abdominal musculature and the backward extension of the hind limbs (Racz et al., 2005). We counted the number of writhing reactions for 30 min after the acetic acid injection.

## 2.3 Carrageenan test

Tissue inflammation produces swelling, long-lasting allodynia and hyperalgesia. To induce tissue inflammation, a small volume (20 µl) of carrageenan (20 mg/ml) was injected subcutaneously into the animal's right hind paw. Tissue inflammation was monitored 1, 4 and 24 h after the injection by measurement of swelling on the affected paw, which was quantified by using a volume meter (TSE System GmbH, Bad Homburg, Germany). Furthermore, we determined the inflammation-induced thermal hyperalgesia 4 and 24 h after injection using the Hargreaves test. The mean withdrawal latency to thermal stimuli was measured by averaging three to five separate trials in the ipsilateral and the contralateral sides.

## 2.4 Formalin test

Male mice were injected with 20 µl of 5% formalin (Appli-Chem, Darmstadt, Germany) into the plantar surface of the right hind paw (Yi and Barr, 1997), the left paw served as control. Formalin injection induces a biphasic pain response, the early phase response (1–5 min), caused by the chemical substance, the formalin and the late phase responses (15–30 min) induced by the inflammatory processes (Fuchs et al., 2011). The total number of pain responses (paw lifting, shaking and licking) was counted on the ipsilateral and contralateral sides. After 90 min, mice were killed by cervical dislocation; the lumbar section of the spinal cord was rapidly removed, shock frozen in 99.5% isopentan (Sigma-Aldrich, Hamburg, Germany) on dry ice and stored at −80°C until use (Bon et al., 2002; Shields et al., 2010).

## 2.5 Partial nerve ligation

To examine the development of neuropathic pain in wild-type and heterozygous animals, a partial nerve ligation (PNL) was performed. Baseline nociceptive responses were determined before the surgery. Under isoflurane (Abbott GmbH & Co. KG Wiesbaden, Germany) anaesthesia, the right sciatic nerve was exposed at the upper thigh level. One-third to one-half of the nerve at mid-thigh level was slightly ligated with a 9–0 silk thread as previously described (Racz et al., 2008) ($n$ = 18 per genotype). Subsequently, the incision was closed in layers. In sham-operated control animals, a similar operation was performed on the right side but without ligation of the sciatic nerve ($n$ = 6 per genotype). Development of mechanical allodynia and thermal hyperalgesia in *Zeb2*$^{+/+}$ and *Zeb2*$^{+/-}$ animals was tested on day 3, 6 and 10 after surgery using the von Frey, the Hargreaves and cold plate tests as previously published (Racz et al., 2008; Jeub et al., 2011).

## 2.6 Immunohistochemistry

DRGs from wild-type and heterozygous animals were dissected, embedded in Tissue-Tek® (Sakure Finetek Europe B.V. Zoeterwoude, The Netherlands) and directly shock frozen in isopentan on dry ice and stored at −80°C until use. DRGs were then sectioned at 9 µm on a Leica CM3050 S cryostate (Leica Microsystems GmbH, Nussloch, Germany). Slices were subsequently fixed in 4% paraformaldehyde for 30 min. Next, slides were washed and then permeabilized in 0.5% Triton X-100 (Sigma-Aldrich Biochemie GmbH, Hamburg, Germany) for 1 h. After blocking in 3% bovine serum albumin, the primary antibody was applied directly on the slices and incubated for 20 h in a wet compartment at 4°C. The following antibody concentrations were used: mouse anti-NeuN (1:500 for 18 h, Millipore, Schwalbach, Germany), rabbit anti-c-Fos (1:5000 for 48 h, Merck, Darmstadt, Germany). A secondary antibody goat-anti-rabbit conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) was used to reveal c-Fos-immunoreactive (IR). We washed the slides three times before mounting in Fluoromount-G™ (SouthernBiotech, Birmingham, AL, USA). Sealing with water varnish prevented sections from drying out.

## 2.7 Image acquisition and analysis

Immunofluorescent pictures were acquired on a Zeiss Axioplan microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and recorded with a monochrome Zeiss Axiocam (Carl Zeiss Microscopy). Image analysis was performed using ImageJ after inversion of the greyscale images. For quantification of c-Fos-IR in the spinal cord, shapes of laminae I/II, III/IV and V/VI were traced according to Watson and Paxinos (Watson and Paxinos, 2009). For statistical analysis, at least seven sections per animal were evaluated for both the c-Fos and NeuN staining.

## 2.8 Electron microscopy

The sciatic nerve was fixed in a solution of 3% glutaraldehyde with HEPES buffer (Sigma-Aldrich Biochemie) (0,1 M pH 7,5) for 24 h, washed for 3 × 10 min with the same buffer, dehydrated with serials of ethanol and embedded in Epon. Ultrathin sections were contrasted using conventional

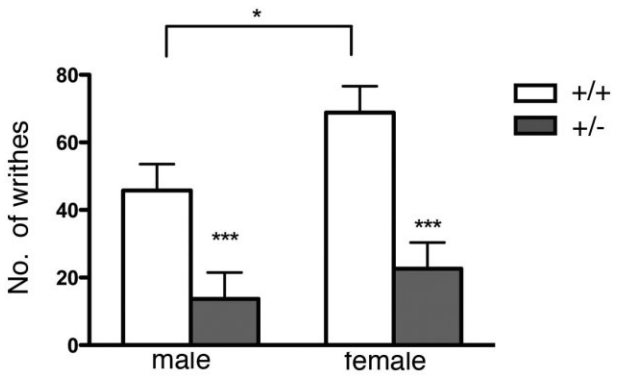


**Figure 1** *Zeb2*$^{+/-}$ mice showed strongly reduced pain responses in the writhing test. Intraperitoneal injection of 0.6% acetic acid in wild-type animals resulted in an increased number of writhes, at which females developed stronger hyperalgesia than male animals. Animals with a heterozygous mutation for *Zeb2* displayed significantly reduced visceral pain. *Zeb2*$^{+/-}$: $n = 10$ male, $n = 10$ female, *Zeb2*$^{+/+}$: $n = 10$ male, $n = 10$ female. Data were analysed with two-way ANOVA and represented as mean ± SD $*p < 0.05$, $***p < 0.001$.

techniques and examined in a Zeiss 900 electron microscope (Carl Zeiss Microscopy) (Schroder et al., 2002). For semi-quantitative analysis, myelinated and unmyelinated fibres were counted on at least eight pictures per section at high magnification (4400x).

## 3. Results

### 3.1 Reduced inflammatory pain in *Zeb2*$^{+/-}$ animals

In the writhing test, *Zeb2*$^{+/-}$ animals displayed significantly fewer abdominal constrictions than *Zeb2*$^{+/+}$ (wild-type, control) littermates [$F_{(1,35)} = 23.76$, $p < 0.001$], indicating a reduced visceral pain sensitivity (Fig. 1). In accordance with other studies, we found a sex difference in this test in wild-type animals, where male mice showed fewer pain responses than females [$F_{(1,16)} = 6.61$, $p = 0.02$] (Chaban, 2012). No gender difference was observed in *Zeb2*$^{+/-}$ animals. Injection of 5% formalin into the hind paw induces two distinct periods of pain. The early, acute phase starts directly after the injection, whereas the late, inflammatory phase is initiated after a transient decline of the acute pain responses. *Zeb2*$^{+/-}$ animals showed significantly reduced pain responses after formalin injection in the early and late phases [$F_{(1,12)} = 8.78$, $p < 0.05$] compared with *Zeb2*$^{+/+}$ animals (Fig. 2A). The noxious input triggered by formalin is conducted via nociceptive C- and Aδ fibres to relay neurons in the dorsal horn of the spinal cord, which are mostly located in the laminae I/II. Activation of these cells triggers the expression of the immediate early gene c-Fos (Hunt et al., 1987), which has emerged as a valuable marker for neuronal activation after noxious stimulation (Harris, 1998). Therefore, we killed *Zeb2*$^{+/-}$ and *Zeb2*$^{+/+}$ animals 90 min after formalin injection, isolated the L4/5 region of the spinal cord and stained transversal sections with a c-Fos antibody (Fig. 2B). c-Fos-IR cells were then counted in laminae I/II, III/IV and V/VI. Formalin treatment induced a significantly higher neuronal activity in laminae I/II [$F_{(1,14)} = 5.05$; $p < 0.001$] in *Zeb2*$^{+/+}$ animals (Fig. 2C). In contrast, we found a significantly reduced c-Fos protein expression in *Zeb2*$^{+/-}$ animals in laminae I/II compared with *Zeb2*$^{+/+}$ littermates. Semiquantitative analysis revealed that the number of c-Fos-IR cells was reduced by 60% in the ipsilateral laminae I/II of the spinal cord in *Zeb2*$^{+/-}$ mice ($p < 0.001$). However, no significant changes were observed in laminae III/IV and V/VI, albeit c-Fos-IR was decreased in *Zeb2*$^{+/-}$ animals.

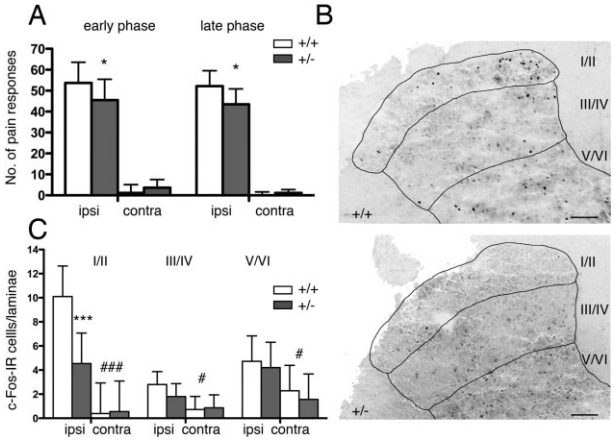


**Figure 2** *Zeb2*$^{+/-}$ animals displayed hypoalgesia and reduced neuronal activity in the dorsal horn after formalin injection. (A) *Zeb2*$^{+/-}$ animals displayed significantly decreased number of pain responses during the early and late phases of the formalin test. *Zeb2*$^{+/-}$: $n = 8$, *Zeb2*$^{+/+}$: $n = 6$. Data were analysed with repeated measurement ANOVA and represented as mean ± SD $*p < 0.05$. (B) *Zeb2*$^{+/-}$ mice showed decreased formalin-induced c-Fos expression in the spinal cord. (C) Quantification of c-Fos-immunoreactive (IR) cells within the laminae I/II showed a significantly decreased neuronal activity in *Zeb2*$^{+/-}$ animals compared with *Zeb2*$^{+/+}$ mice (*Zeb2*$^{+/+}$ $n = 4$; *Zeb2*$^{+/-}$ $n = 5$). No significant differences were observed in laminae III/IV and V/VI. Data were analysed with factorial ANOVA and represented as mean ± SD *represent comparison between genotypes; #indicate comparison between contralateral and ipsilateral sides. $***p < 0.001$; $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$. ipsi = injected side; contra = non-injected, contralateral side; IR, immunoreactive. Scale bars represent 100 μm.

### 3.2 Hyperalgesia and allodynia are not influenced by mutations in *Zeb2*

Next, we characterized the effect of the heterozygous deletion of *Zeb2* on the development of paw oedema in the carrageenan test. The mutation had no effect on the development of thermal hyperalgesia tested 4 and 24 h after injection (Fig. 3A). After 1, 4 and 24 h, $Zeb2^{+/+}$ and $Zeb2^{+/-}$ animals displayed a prominent swelling in the injected paw (Fig. 3B). In both genotypes, carrageenan treatment decreased the withdrawal latency significantly by 60%.

We also assessed mechanical and thermal allodynia and thermal hyperalgesia in a neuropathic pain model produced by partial ligation of the sciatic nerve. For mechanical allodynia, we used the von Frey filament test (Fig. 4A). Baseline responses of $Zeb2^{+/-}$ and $Zeb2^{+/+}$ littermates were similar, and sham operation did not produce any modification for the nociceptive responses in any of the genotypes. Mechanical allodynia in the operated side after nerve injury was revealed by the reduced reaction latency [$F_{(1, 44)} = 24.1$, $p < 0.001$]. We did not find any difference between $Zeb2^{+/+}$ and $Zeb2^{+/-}$ animals [$F_{(1, 44)} = 0.98$, $p$ = n.s.]. For thermal hyperalgesia, we used the Hargreaves test (Fig. 4B), and we determined the same basal responses as published earlier (Jeub et al., 2011). $Zeb2^{+/+}$ and $Zeb2^{+/-}$ animals presented the same hyperalgesia [PNL: $F_{(1, 17)} = 66.12$, $p < 0.001$; strain: $F_{(1, 17)} = 0.624$, $p$ = n.s.]. Additionally, we assessed thermal allodynia using the cold plate test (Fig. 4C). Similar to the previous tests, we could only detect an effect of the surgery but not of the genotype [PNL: $F_{(1, 17)} = 28.9$, $p < 0.001$; strain: $F_{(1, 17)} = 0.01$, $p$ = n.s.].

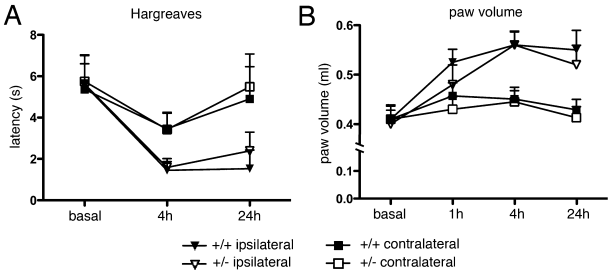


**Figure 3** $Zeb2^{+/-}$ mice showed comparable paw swelling after injection of carrageenan to the $Zeb2^{+/+}$. (A) Both $Zeb2^{+/+}$ and $Zeb2^{+/-}$ animals showed increased thermal hyperalgesia on the injected, ipsilateral side 4 and 24h after injection. (B) One, 4 and 24 h after intraplantar injection of carrageenan, $Zeb2^{+/+}$ and $Zeb2^{+/-}$ animals showed a significant increase in paw volume. $Zeb2^{+/-}$: $n = 8$, $Zeb2^{+/+}$: $n = 7$. Data were analysed with repeated measurement ANOVA and represented as mean ± SD ***indicates comparison between contralateral and ipsilateral reactions and latencies at different time points after injection. ***$p < 0.001$. Ipsilateral = injected side; contralateral = non-injected.

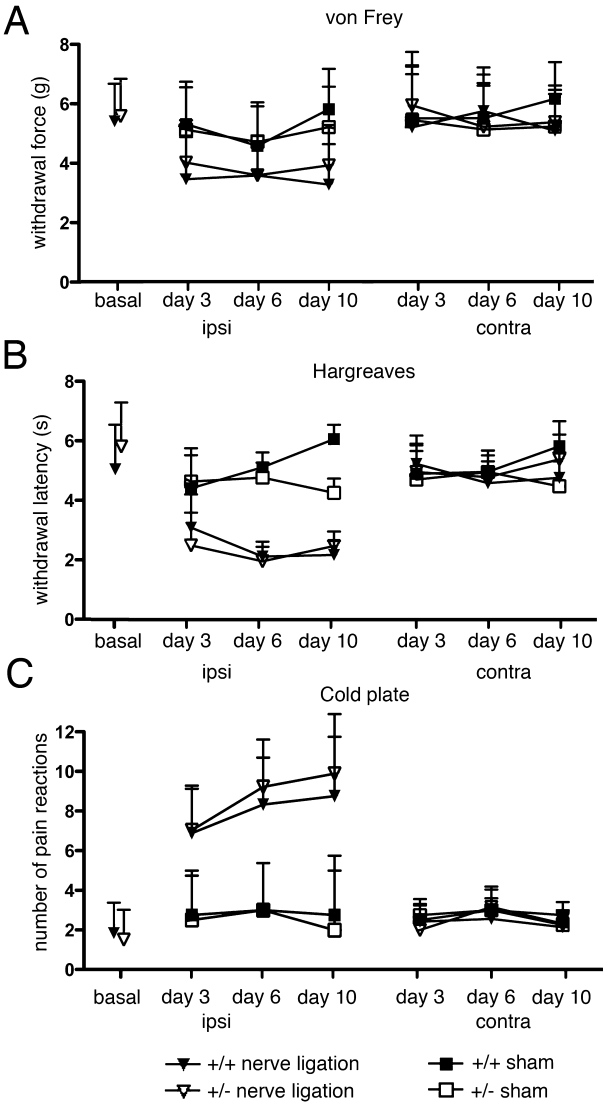


**Figure 4** $Zeb2^{+/-}$ and $Zeb2^{+/+}$ mice showed similar neuropathic pain responses. Partial nerve ligation was performed to induce neuropathic pain in $Zeb2^{+/-}$ and $Zeb2^{+/+}$ animals. Mice were tested on the ipsilateral, operated and the contralateral, non-operated side, for mechanical allodynia using the von Frey model (A), for thermal hyperalgesia using the Hargreaves test (B), for thermal allodynia using the cold plate test (C). Thermal hyperalgesia and mechanical allodynia are revealed by decreased withdrawal threshold of the affected (ipsilateral) paw. Thermal allodynia is reflected in higher number of pain reactions on the cold plate. The elevated pain reactions were significant in both genotypes. (von Frey test: $n = 18$ in both genotype, Hargreaves and cold plate tests: $n = 8$ per group) Data were analysed with repeated measures ANOVA and represented as mean ± SD ***$p < 0.001$.

### 3.3 Morphology of DRG neurons and sciatic nerve

To determine if the morphology of DRG neurons is affected in $Zeb2^{+/-}$ mice, we stained serial DRG sections

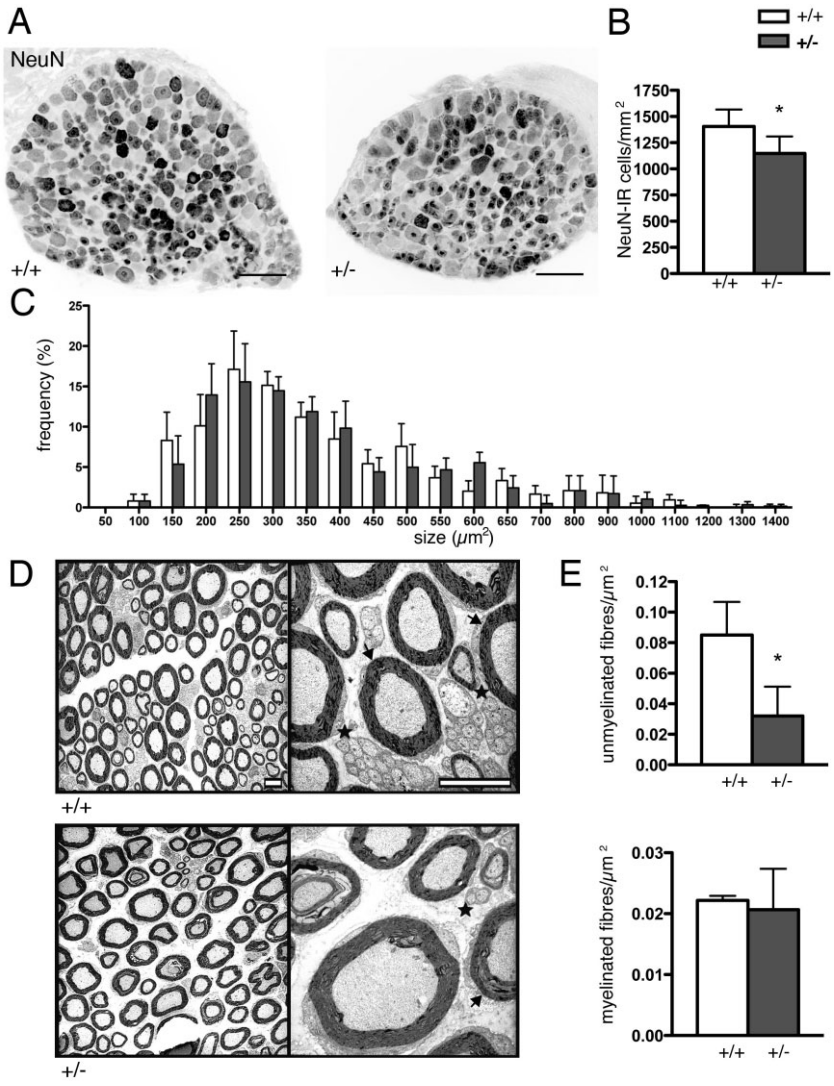


**Figure 5** *Zeb2*$^{+/-}$ animals displayed reduced density of neurons and unmyelinated fibres. (A) Representative images of dorsal root ganglia (DRGs) neurons as revealed by NeuN immunostaining. (B) We found significantly fewer NeuN expressing cells in DRGs of *Zeb2*$^{+/-}$ mice (*Zeb2*$^{+/+}$: $n = 5$; *Zeb2*$^{+/-}$: $n = 6$ animals per group), (C) whereas the overall distribution of neuronal cell sizes was not significantly different between *Zeb2*$^{+/+}$ and *Zeb2*$^{+/-}$ littermates ($n = 3$ animals per group). Frequency (%) represents the percental size distribution of neurons normalized to the total number of counted neurons per animal. Data were analysed with one-way ANOVA and represented as mean value ± SD; *$p < 0.05$. Scale bars represent 100 μm. (D) Representative electron micrograph of the *sciatic nerve* revealing myelinated (arrowheads) and unmyelinated (stars) nerve fibres. In wild-type mice, we found more unmyelinated fibres compared with heterozygous animals. Scale bars represent 5 μm. (E) Semi-quantitative analysis of unmyelinated and myelinated fibres in the sciatic nerve ($n = 3$ per group). Data were analysed with one-way ANOVA and represented as mean value ± SD *$p < 0.05$.

with a neuron-specific antibody and evaluated neuronal cell numbers and size (Fig. 5A). We found a reduction of 18% in neuronal cell density in *Zeb2*$^{+/-}$ mice [$F_{(1,9)} = 6.77$; $p < 0.05$] (Fig. 5B) but no significant difference in the overall distribution of the cell size (Fig. 5C) accordingly to our previous publication (Jeub et al., 2011). However, a detailed analysis of the sciatic nerve by electron microscopy (Fig. 5D-E) showed a marked reduction of unmyelinated fibres in *Zeb2*$^{+/-}$ animals.

## 4. Discussion

Our data demonstrate that inactivation of one allele of *Zeb2* in mice leads to a reduced pain sensitivity in the writhing and formalin tests. However, it neither affected the development of thermal hyperalgesia after carrageenan injections nor mechanical allodynia after peripheral nerve injury. Evaluation of c-Fos expression after the formalin test revealed a decreased neuronal activity in the spinal laminae I/II. Particularly, this lamina contains second order neurons, which receive input from polymodal C- and Aδ fibres (Heise and Kayalioglu, 2009). The reduced c-Fos activation suggests that the nociceptive input from the DRG neurons is decreased in *Zeb2*$^{+/-}$ animals. In accordance with this, our previous results demonstrated that *Zeb2*$^{+/-}$ animals show longer reaction latencies towards acute thermal but not towards mechanical stimuli (Jeub et al., 2011). One possible explanation for this phenotype is a deficit in a particular subset of nociceptors due to the mutation. Aδ fibres play an important role in acute pain sensation. Because they are polymodal, they contain subpopulations with varying sen-

sitivity to heat and mechanical stimuli (Meyer et al., 2006). It is possible that those with a low heat and a high mechanical threshold (Aδ type II) are impaired in number and/or function.

The role of *Zeb2* has been well studied during embryogenesis and in early post-natal development. Homozygous deletion of *Zeb2* leads to defects in the development of the enteric and autonomic nervous system and deficits in the induction of neural crest cells (Van de Putte et al., 2003, 2007). The neural crest is a transient multipotent cell population that develops into various tissues, including DRG neurons and satellite glial cells (Lawson and Biscoe, 1979; Hatten and Heintz, 1999). Therefore, we considered the possibility that *Zeb2* is also involved in the differentiation of nociceptors, which develop from neural crest cells (Lawson and Biscoe, 1979; Maro et al., 2004). Indeed, during tissue preparation, we realized that the DRGs of $Zeb2^{+/-}$ animals were smaller compared with those of their littermates. Analysis of NeuN-stained neurons in consecutive DRG sections supported our hypothesis that *Zeb2* affects the differentiation of DRG neurons. The fact that we found fewer neurons in the DRG of $Zeb2^{+/-}$ animals indicated a reduced sensory innervation of peripheral tissues. This finding was underscored by our electron microscopic data, which showed a lower number of unmyelinated C-fibres in the sciatic nerve of heterozygous animals. This phenotype is reminiscent to what has been described for a mouse strain lacking both alleles of *Zeb2* specifically in the neural crest. During embryonic development, these conditional knockout mice showed a reduced number of neurons in the sympathetic ganglia and a more loosely organization of the nerve cells (Van de Putte et al., 2007). It has been suggested that neural crest-derived sympatho-adrenal precursor cells migrate correctly but proliferate abnormally. Neural crest-specific $Zeb2^{-/-}$ knockouts apparently also show a deficit in DRG size, although this has not been studied in detail (Van de Putte et al., 2007). It may be interesting to investigate in future studies if the reduced neuronal density in heterozygous *Zeb2* knockout mice resulted from decreased proliferation, increased apoptosis, deficits in cell migration or other developmental deficits during the differentiation and specification of sensory neurons (Miquelajauregui et al., 2007). DRG neurons develop from neural crest cells in at least two and according to some three consecutive waves (Lawson and Biscoe, 1979). First, medium-to-large-sized proprioceptive and low-threshold mechanoreceptive DRG neurons arise, which express the receptor tyrosine kinase trkB and trkC (Jones et al., 1994; Farinas et al., 1996). During the later wave of neurogenesis, mostly small substance P (SP-) or calcitonin gene-related protein (CGRP)-synthesizing nociceptive neurons are born, which depend on trkA signalling (Crowley et al., 1994). About a half of the trkA positive cells differentiate post-natally into 'non-peptidergic' nociceptors, which do not produce the neuropeptides SP or CGRP but express the tyrosine kinase Ret, the receptor for the glial-derived neurotrophic factor (GDNF) (Bennett et al., 1996). Genetic disruption of neurotrophin signalling pathways leads to a severe reduction of DRG cell populations (Crowley et al., 1994; Jones et al., 1994; Farinas et al., 1996). In this context, it is important to note that *Zeb2* directly binds to the promotor region of neurotrophin-3 in the embryonic cortex, thereby regulating (in fact repressing) its expression there (Seuntjens et al., 2009). It is conceivable that neurotrophin expression is also affected by the heterozygous deletion of *Zeb2* in primary sensory DRG neurons, but here leading to a reduced nociceptive innervation in the periphery.

We previously showed that heterozygous deletion of *Zeb2* altered the electrophysiological responses of DRG neurons. Specifically, Transient Receptor Potential Vanilloid 1 (TRPV1) positive primary afferents showed a significantly reduced frequency in action potential firing upon electrical stimulation (Jeub et al., 2011). In parallel, the neurons showed increased persistent sodium currents and a reduction of delayed rectifier potassium currents indicating that *Zeb2* also controls the transduction properties of nociceptive DRG neurons possibly by transcriptional regulation of ion channels. Interestingly, these molecular and morphological changes do not interfere with the development of hyperalgesia in the carrageenan test and after partial nerve ligation. Conditions of chronic pain are often accompanied by a phenotypic switch in DRG neurons. Aβ-fibres appear to play an important role in this process, resulting in an increased mechanical allodynia and the development of spontaneous ectopic activity (Liu et al., 2000; Xu and Zhao, 2001; Devor, 2006). Further, non-peptidergic neurons are also shown to be involved in contributing to the development of neuropathic pain. It has been shown that treatment with GDNF after nerve injury effectively reduced mechanical allodynia and thermal hyperalgesia (Boucher et al., 2000). More recently, it was demonstrated that spinal nerve ligation leads to an increased expression of TRPV1 on non-peptidergic C-fibres, which was paralleled with a gain in heat sensitivity (Vilceanu et al., 2010). Heterozygous *Zeb2* deletion did not affect chronic pain conditions. It is

thus unlikely that this transcription factor is essential for phenotypic changes of DGR neurons.

However, our results clearly show that *Zeb2* affects acute nociception, including formalin- and proton-induced pain, first by reduced innervation of the periphery, and second by altered ion channel expression on TRPV-1-positive polymodal nociceptors (Jeub et al., 2011). In the context of MWS, this may indicate that the under-reaction to pain, which is reported for some of the patients already (Evans et al., 2012), results from a reduced responsivity to nociceptive stimulation rather than an inability to communicate discomfort.

## Author contributions

B.P., I.R., A.Z. designed experiments and wrote manuscript.

B.P., I.R., A.M., D.M., K.T. performed experiments.

T.V.dP., E.S. and D.H. generated the mouse, article revision.

M.J, V.G-D., H.F., M.H.dA., H.B. contributed to phenotyping of the mouse and article revision.

All authors discussed the results and commented on the manuscript.

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