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Mapping of PRRXL1 functional domains

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Mapping of PRRXL1 functional domains

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ABSTRACT

PRRXL1 is a paired-like homeodomain transcription factor expressed in nociceptive neurons of dorsal root ganglia and dorsal spinal cord. Evidence shows that PRRXL1 is crucial in the establishment and maintenance of the nociceptive system, as Prrxl1$^{-/-}$ mice present neuronal loss, reduced nociception and failure to thrive. In this study it is shown that PRRXL1 is highly phosphorylated *in vivo*, and that its multiple band pattern on electrophoretic analysis is the result of different phosphorylation states. These phosphorylations evolve along spinal cord and dorsal root ganglia development from a higher to a lower phosphorylated state, and are mapped to aminoacid regions 1-143 and 227-263. PRRXL1 also displays a conformation, a dimerization and a repressor domain, which altogether act in concert to modulate its transcriptional activity. Phosphorylation is therefore proposed as a mechanism for regulating PRRXL1 function and conformation during nociceptive system development.

Keywords: Development; nociceptive system; phosphorylation; Prrxl1

ABBREVIATIONS

CIAP: calf intestinal alkaline phosphatase
DRG: dorsal root ganglion
dSC: dorsal spinal cord
IEF: isoelectric focusing
IMAC: immobilized metal affinity chromatography
NLS: nuclear localization sequence
OAR: *otp*, *aristaless* and *rax*
R*f*: electrophoretic mobility
WB: western blotting

INTRODUCTION

Pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" [1]. Although the functional maturation of higher brain centres is needed for complete pain awareness and response, that would not be possible without the existence of a properly established spinal nociceptive circuitry [2]. This circuitry is important for the adequate relay of sensory information from the peripheral tissues to the brain, and its embryonic development is regulated by the combinatory expression of transcription factors, which specify distinct neuronal populations. The expression of morphogens and their receptors, basic Helix-loop-Helix transcription factors and, later, homeodomain transcription factors shapes the dorsal spinal cord (dSC) into its laminated cellular pattern and divides the dorsal root ganglion (DRG) into three main neuronal populations [3,2]. The ensuing DRG/spinal nociceptive circuit is therefore composed by small diameter primary afferent neurons that project to the superficial *laminae* of the dSC.

The molecular mechanisms responsible for the proper establishment of these neuronal connections are not yet fully understood. Paired-related homeobox protein-like 1, or *Prrxl1* (also known as *Drg11*), is a gene encoding for a transcription factor that is involved in the establishment and maintenance of the DRG-dSC nociceptive circuitry during embryonic development [4]. It is expressed in the DRG, dSC, cranial sensory ganglia and brainstem sensory nuclei [5]. *Prrxl1*$^{-/-}$ mouse embryos exhibit spatio-temporal abnormalities in the patterning of sensory afferent fibre projections to the dSC, as well as loss of dorsal horn neurons. Post-natally, *Prrxl1* null mutant mice present significantly attenuated sensitivity to noxious and thermal stimuli, with reduced sensitivity to mechanical stimulation and chemical nociception. The apparent absence of alterations in motor function and locomotion suggests that proprioception and mechanical innocuous processing are not influenced by PRRXL1 [4]. At the cellular level, PRRXL1 seems to be important for the survival of peptidergic and non-peptidergic small DRG neurons, as opposed to large DRG neurons [6]. *Prrxl1* expression markedly decreases after birth, being residual in adult life. However, post-natal expression is increased in inflammatory, but not neuropathic pain, at the expense of peptidergic and non-peptidergic small DRG neurons [7].

Although the role of PRRXL1 in the development of the DRG/dSC circuit has been well established, the mechanisms underlying the modulation of PRRXL1 transcription activity remain unstudied. PRRXL1 western blotting (WB) analysis of SC and DRG extracts revealed a pattern of multiple bands [5], which may indicate the occurrence of post-translational modifications such as phosphorylation [8]. In fact, more than 400 types of post-translational modifications can occur, with the most common occurring in eukaryotic cells being phosphorylation [9]. Protein phosphorylation is a major mechanism by which transcription factor activity is regulated. Moreover, it seems to be the post-translational modification of choice when rapid modulation of transcription factor activity is required [10]. Protein phosphorylation is known to regulate several cellular processes, namely cell growth and division, differentiation, signal transduction and gene expression [11].

The present study shows that PRRXL1 band pattern is due to multiple phosphorylation states that vary along DRG and dSC development. Analysis of several PRRXL1 truncated forms suggests that PRRXL1 has two phosphorylation clusters, with an overall number of at least 6 phosphorylated residues. These phosphorylations seem to be responsible for changes in protein conformation and thereby transcriptional activity. Overall, this work comes a step closer to

uncovering the molecular mechanisms underlying the regulation of PRRXL1 function in the establishment of the nociceptive circuitry.

MATERIALS AND METHODS

Animal care and Tissue Harvesting - NMRI mice were bred and fed *ad libitum* with a standard diet and housed at the IBMC animal facility under temperature- and light-controlled conditions. Embryonic day 0.5 (E0.5) was considered to be the midday of the vaginal plug. The animals were euthanized (isoflurane anesthesia followed by cervical dislocation) and embryos surgically removed. The SC and DRG were collected and the SC dissected into their dorsal and ventral moieties, except for E10.5 embryos, where the prospective SC was used. Experiments complied with the European Community Council Directive (86/609/EEC) and the animal ethics guidelines at IBMC. The Portuguese Veterinary Ethics Committee approved all animal manipulations.

1 dimensional (1D) and 2 dimensional (2D)-PAGE sample preparation - dSC nuclear lysates for 1D-PAGE were obtained as previously described [5]. dSC and DRG were homogeneized in 2D lysis buffer (20 mM phosphate buffer pH 8.0, 0.1% Triton X-100) supplemented with phosphatase and protease inhibitors (Sigma-Aldrich) and Benzonase nuclease (Novagen), sonicated and cleared by centrifugation. Isoelectric focusing (IEF) was performed on a Protean i12 Cell (Bio-Rad) using 11 cm immobilized pH gradient strips pH 3-10 non-linear (Bio-Rad) for 38.000 VH, held at 750 V and frozen until processing. The low-bis SDS-Polyacrilamide gels (10-16%T/1.35%C) were ran, and WB was performed using standard protocols. Rabbit anti-HA (Invitrogen #715500), homemade rabbit anti-PRRXL1 [5], mouse anti-FLAG M2 (Sigma-Aldrich #F1804), mouse anti-α-tubulin (Sigma-Aldrich #T5168) and rabbit anti-Histone H3 (Abcam #ab1791) were used as primary antibodies. Appropriate HRP conjugated secondary antibodies (Jackson Immunoresearch Europe) or Clean-Blot IP Detection Reagent (Pierce) were used. All quantitative blot analyses were performed using ImageLab v4.1 (Bio-Rad Laboratories).

Dephosphorylation assays - Samples homogeneized in dephosphorylation buffer [12] were incubated with calf intestinal alkaline phosphatase (CIAP) (Invitrogen) for 2 h at 37ºC. A competitive inhibitor, 20 mM Na_2HPO_4, was added to a replicate reaction mix as a control. IEF samples were prepared using ReadyPrep™ 2-D Cleanup Kit (Bio-Rad).

Ga(III)- immobilized metal affinity chromatography (IMAC) - The matrix was generated using IMAC-Select Affinity gel (Sigma-Aldrich) and $GaCl_3$. The gel was washed in 50 mM EDTA/1 M NaCl and charged with 100 mM $GaCl_3$ in 100 mM acetic acid. It was washed with 5% acetic acid followed by 0.5 M NaCl and H_2O washes. $GaCl_3$ was not added to the control matrix. Nuclei were lysed in IMAC lysis buffer (15 mM MES/NaOH pH 5.5, 0.5 M NaCl, 1% NP-40, protease inhibitor cocktail). Matrix was equilibrated in IMAC buffer (50 mM MES/NaOH pH5.5, 0.5 M NaCl, 1% NP-40) and extracts (0.1 µg/µL) were incubated for 2 h at 4ºC. After washing, elution was performed by adding 0.2 M Phosphate buffer pH 8.0 [modified from 13]. The input and collected fractions were TCA-precipitated prior to analysis.

GST- PRRXL1 generation and purification - BL21 *E. coli* transformed with pGEX-4T3-*Prrxl1* were induced, lysed (PBS, 0.5% Triton, 1 mM DTT and inhibitor cocktails), incubated 2 h at 20ºC with a glutathione-cellulose matrix (Bioline) and eluted with thrombin (Novagen). The SDS-PAGE gel of the eluate was reverse stained with Zn^{2+} [14] and the PRRXL1 band was

excised. PRRXL1 was eluted from the gel by diffusion overnight at 4ºC in 0.01% SDS/20 mM Tris-HCl pH 7.5, TCA–precipitated, ressuspended in 2% SDS and dialyzed against a 10 mM Tris-HCl pH 7.5, 0.01% n-octyl-β-D-glucoside solution, to allow renaturation of the fusion protein.

Prrxl1 constructs - Full-length and N/C-terminal truncations of *Prrxl1* (EU670677.1) were amplified from mouse E14.5 SC cDNA and cloned into pCDNA3.3-TOPO TA (Invitrogen). The HA- and FLAG-tag were added to the primers (see Supplementary Table 1). All constructs were sequenced.

Cell culture - ND7/23 and HeLa cell lines were cultured at 37ºC/5ºCO_2 in DMEM (Gibco) containing 10% FBS (Gibco) and 50 U/ml penicillin and streptomycin (Gibco). Transfection was performed using Lipofectamine 2000 (Invitrogen) and cells were harvested 24 h later.

Luciferase reporter assays – 48 h after transfecting ND7/23 or HeLa cells with different constructs of PRRXL1, pCMVβ (Clontech) and pGL3 (Promega) containing Firefly Luciferase under the control of the *Rgmb* promoter sequence [15], cells were resuspended in 50 µl of lysis buffer (Promega) and the protein extract cleared by centrifugation. 5 µl were mixed with the luciferase reagent (Promega) and luminescence measured by an Infinite M200 plate reader (Tecan). Transfection efficiency was normalized by assessing β-gal activity using ONPG (Sigma-Aldrich) as substrate and read at 420 nm.

Cyanogen bromide (CNBr) cleavage assay - ND7/23 cells overexpressing PRRXL1-HA, lysed in a 1% Triton X-100, 0.1% SDS buffer were incubated for 20 or 90 min with 10 volumes of a 100 mg/mL CNBr solution in 70% formic acid. Afterwards, 10 volumes of 10% TCA in acetone were added, incubated for 1 h at -80ºC, centrifuged at 21,100*g* for 30 min and washed twice in acetone. Precipitate was resuspended in adequate downstream buffer [modified from 16].

DNA-protein interaction studies – 50 µg of transfected ND7/23 cell extracts were incubated overnight at 4ºC with 150 ng of biotinylated *Rgmb* promotor sequence [15] (PCR-generated using biotinylated primers) and 1 µg of Salmon sperm DNA (Sigma Aldrich), in a volume of 100 µL of wash buffer (TBS, 5% BSA, 0.05% Tween-20). Control reactions omitted the DNA probes. The reaction mix was incubated for 2 h at room temperature in NeutrAvidin coated 96-well plates (Pierce) and washed. Anti-HA antibody 1/1000 was added for 3 h at room temperature, followed by washing and 1 h incubation with a 1/5000 dilution of HRP-conjugated goat anti-rabbit antibody. WestPico Chemiluminescent substrate (Pierce) was added and luminescence measured after 2 min on an Infinite M200 reader (Tecan).

Ferguson Plot - Ferguson plot analysis can distinguish a change in the shape of a protein from a change in the charge. By running ND7/23 extracts overexpressing PRRXL1-HA in SDS-PAGE at various concentrations of acrylamide (10 to 16%, w/v), the logarithm of the electrophoretic mobility (R*f*) was plotted *versus* the acrylamide concentration. The slope and y-axis intercept depend on the electrical charge of the protein and on the shape, defined as the Stokes radius (effective hydrated radius of the protein-SDS complex). Proteins differing only in the conformation give different slopes with a common y-axis intercept whereas proteins differing only in the electrical charge have identical slopes with different y axis intercepts [17,18]. Linear regression was performed in Microsoft Excel 2010®.

Limited tryptic digestion - ND7/23 cells overexpressing *Prrxl1* lysed in TBS-0.1%, Triton X-100 were incubated with TPCK-treated trypsin (Sigma-Aldrich #T1426) at ratios of 1:100 to 1:400 (w/w). The reactions were performed in 0.05% BSA, 50 mM Tris-HCl pH 8.0, 20 mM

$CaCl_2$ and 0.5 mM DTT at 37ºC during 15 min, after which 5 mM AEBSF and sample buffer were added. Peptides were resolved by 16% SDS-PAGE.

Cycloheximide time-course - ND7/23 cells were reverse-transfected with pCDNA3.1-*Prrxl1*. After 12 h, the cells were incubated with cycloheximide (100 µg/ml) for 1 to 6 h and the lysates analysed by WB.

Sub-cellular fractionation of Prrxl1 *constructs* – ND7/23 cells transfected with different *Prrxl1* constructs were lysed in nuclear fractionation buffer (0.25 M sucrose, 20 mM Tris-HCl pH 7.4, 0.1% Triton X-100 and protease and phosphatase inhibitors), gently homogeneized and centrifuged at 1000*g* for 10 min at 4ºC. Equivalent amounts of total, cytosolic and nuclear lysate were loaded.

Immunocytochemistry of constructs – ND7/23 cells transfected with different *Prrxl1* constructs were fixed in 4% PFA in PBS, permeabilized using 1% Triton X-100 in PBS, blocked with 10% NGS in PBS/0.1% Triton and incubated overnight at 4ºC with anti-PRRXL1. Alexa 594 conjugated goat anti-rabbit IgG (Molecular Probes) was used as secondary antibody and DAPI was used as a nuclear counterstain.

Co-immunoprecipitation - ND7/23 cells were co-transfected with PRRXL1-FLAG and several HA-PRRXL1 truncated constructs. Lysates in co-immunoprecipitation buffer (TBS, 0.1% Triton X-100, 10% glycerol and protease and phosphatase inhibitors) were immunoprecipitated using ANTI-FLAG M2 Magnetic Beads (Sigma-Aldrich) and eluted in Sample Buffer.

Statistical analysis – Students t-test was used and the *p*-values indicated result from at least 3 independent experiments. All error bars represent standard deviation.

RESULTS

PRRXL1 displays a delayed electrophoretic migration due to phosphorylation

PRRXL1 WB analysis showed the presence of multiple bands [5] that are suggestive of post-translational modifications [8,19]. This is further supported by the fact that PRRXL1 expressed in *Escherichia coli* displayed a single band, when compared to E14.5, which displayed a higher molecular-weight duplet (fig. 1a). As the most common post-translational modification in eukaryote organisms is phosphorylation [9], it was hypothesized that the PRRXL1 band pattern could be due to phosphorylation. Therefore, nuclear extracts of dSC from E14.5 embryos were treated with CIAP. The phosphatase treatment reproducibly eliminated the alteration in migration, revealing an increasing amount of dephosphorylated PRRXL1 (lower molecular-weight band) with increasing units of CIAP. The addition of a competitive inhibitor (Na_2HPO_4) prevented this effect, maintaining PRRXL1 band pattern (fig. 1b). Thus, phosphorylation is responsible for the delayed migration of the observed PRRXL1 forms. To further reinforce this observation, E14.5 and post-natal day 0 (P0) dSC nuclear extracts were subjected to Ga(III) IMAC, a resin that binds phosphoproteins [13]. These ages were chosen because they are representative of different PRRXL1 band patterns. As depicted in figure 1c, the E14.5 and P0 eluted fractions displayed an enrichment of the upper bands, and only the lower bands (the more dephosphorylated bands) were detected in the unbound fraction (flowthrough). While providing an important internal control, this demonstrates that the multiple band pattern represents a gradient of differentially phosphorylated forms, with higher phosphorylation states in the upper bands. This also explains the absence of dephosphorylated PRRXL1 in dSC E14.5

incubated with Ga(III), as PRRXL1 expressed in this age displays only highly phosphorylated forms.

The ND7/23 cells are a hybrid cell line derived from neuroblastoma and P0 DRG neurons that displays sensory neuron-like properties [20]. It also endogenously expresses PRRXL1, although at low levels [21]. Lysates of ND7/23 cells overexpressing *Prrxl1* were incubated with CIAP and resolved by SDS-PAGE (fig. 1d). Untreated PRRXL1 displayed a multiple band pattern similar to P0 DRG. As expected, incubation of PRRXL1 with CIAP abrogated upper bands, and Ga(III)-IMAC purification confirmed an enrichment of the upper bands (fig. 1e). Therefore, the ND7/23 cell line is a suitable model for further study of PRRXL1 phosphorylations.

PRRXL1 acquires distinct phosphorylation states along development

When mouse dSC and DRG protein extracts from different developmental stages are resolved by WB, a differential multiple band pattern can be observed (fig. 2a). Overall, four bands can be identified, henceforth referred to as band 1 to 4 from the slowest to the fastest migrating band. At early developmental ages (E10.5 to E14.5), when neuronal specification and migration is occurring [3], only the two upper bands (bands 1 and 2) were detected both in the dSC and in the DRG (fig. 2a). From E16.5 onwards, a developmental period where neurons have migrated and are differentiating [3], there is a progressive change of PRRXL1 upper band toward lower bands (bands 3 and 4). However, while in the dSC there is loss of the upper migrating band (bands 1 and 2) from E16.5 onwards, in the DRG, the upper bands fade only after birth, while band 3 increases at P14 and P21. This differential evolution of phosphorylation patterns in the dSC and in the DRG along nociceptive system development suggests that PRRXL1 plays a specific role in each tissue.

To improve the resolution of our analysis, several protein extracts were subjected to 2D electrophoresis, a technique combining IEF followed by SDS-PAGE (fig. 2b). The IEF separates proteins according to their isoelectric point (p*I*). It can detect individual phosphorylated forms, since each additional phosphate increases the negative charge of the protein, thus causing a shift in p*I* [22]. Therefore, by using IEF, one can monitor the phosphorylation state of a protein as indicated by the shifts in the p*I*. For this analysis, samples from E14.5, E16.5, P0 and P14 mice were used, as these developmental ages seem representative of all PRRXL1 phosphorylation states. The 2D electrophoresis resolved PRRXL1 into a detectable maximum of 7 different spots, which drift towards the anode (fig. 2b). The number of phosphorylated residues for each spot was determined by considering the predicted shift provided by each phosphorylation [23], the expected position in the 3-10 pH gradient and a dephosphorylated E14.5 dSC extract (fig. 2b, CIAP control), where only one spot is seen in the region of PRRXL1 theoretical p*I* (8.74). Therefore, each spot corresponds to different PRRXL1 phosphorylated forms.

In accordance to the observation that PRRXL1 phosphorylated forms vary along development, it is evident that the relative abundance of each spot also changed, and differed between samples. When comparing E14.5 with E16.5 and P0 dSC samples, the intensity of two spots (arrows in fig. 2b, corresponding to 2 and 4 phosphorylated residues) decreased from E14.5 to E16.5 and was undetectable at P0. These spots are part of the upper bands from the 1D analysis (fig. 2a/b, diagrams). On the other hand, the lower bands were only detected at post-natal ages. In the dSC, band 4 is the only one of the lower duplet detected in 1D SDS-PAGE (fig. 2a), giving rise to a spot that migrates in a region of the immobilized pH gradient strip corresponding to one phosphorylated residue (fig. 2b). The PRRXL1 expressed in ND7/23 cells,

however, displayed spots corresponding to 6 phosphorylated residues, and is most similar to the spot pattern of dSC P0. The progressive disappearance of particular spots explains the previous finding that, as development progresses, PRRXL1 tends to exist in a less phosphorylated state. It also suggests that specific phosphorylation states are favoured over others at different stages, and may be mutually interdependent.

PRRXL1 acquires phospho-dependent conformational changes

The fact that the SDS-PAGE resolved phosphorylated PRRXL1 forms with the same isoelectric point (and hence the same number of phosphorylated sites) but with different electrophoretic mobility (fig. 2b, ND7/23) suggests that the electrostatic charge conferred by the presence of multiple phosphate groups is not the sole responsible for PRRXL1 multiple band pattern. This mobility shift could in fact be the result of different protein conformations that rely on phosphorylation at key residues [8].

To further address this issue, the phosphorylation-induced gel shift was studied using the Ferguson plot approach [17,18], by carrying out SDS-PAGE at different acrylamide concentrations and plotting the log(R*f*) as a function of acrylamide concentration (Table 1). The R*f* is proportional to the net charge of the protein and inversely proportional to its Stokes radius. If the mobility decrease was caused only by an alteration in the charge due to phosphorylation, it would be independent of the acrylamide concentration (for further details please refer to the Materials and Methods section). However, the difference in the slopes of the R*f* of the lower (bands 3/4) *versus* the higher (bands 1/2) phosphorylated state of PRRXL1 suggests that phosphorylation causes a change in protein Stokes radius and thus in protein structure. In fact, there is an R*f* decrease with decreasing pore size. This supports the previous hypothesis that PRRXL1 suffers conformational changes, likely induced by phosphorylation.

To further support this observation, the susceptibility of PRRXL1 bands to limited tryptic digestion was used as a conformational probe. ND7/23 extracts overexpressing *Prrxl1* were incubated with different amounts of trypsin and the signal intensity of bands 1/2 and 3/4 was estimated (fig. 3a). The fast migrating bands (bands 3/4) correspond to a less phosphorylated status and were more resistant to enzymatic digestion than the more phosphorylated bands (bands 1/2), whose expression diminishes with increasing trypsin amounts. This result suggests that phosphorylation favours a local conformational change that at least partially alters PRRXL1 structure, with increased exposure of more proteolitically sensitive residues. Furthermore, when ND7/23 cells overexpressing *Prrxl1* were treated with cycloheximide (which inhibits protein synthesis by blocking translational elongation; fig. 3b), there was a decrease of the upper bands' level over time, in comparison with the lower bands. These observations suggest that highly phosphorylated PRRXL1 is less stable.

PRRXL1 homodimerizes and has two phosphorylation clusters

Aiming at identifying the main PRRXL1 phosphorylated regions, several truncated forms of HA-PRRXL1 were generated (fig. 4a) and the nuclear translocation of the constructs was assessed (fig. 4b/c). It has been suggested that paired-like homeodomain transcription factors contain a bipartite nuclear localization sequence (NLS) motif flanking the homeodomain region [24]. In fact, all PRRXL1 truncations containing the homeodomain/NLS sequence translocated to the nucleus, while versions with deletion in this region remained in the cytoplasm. However, a small amount of $PRRXL1_{107\text{-}263}$ was still translocated to the nucleus despite the deletion of the homeodomain, while all $PRRXL1_{189\text{-}263}$ remained in the cytosol (fig. 4c). This could be explained by dimerization of the truncated form with endogenous PRRXL1

or other transcription factors, as paired-like homeodomain proteins usually act as dimers to form a functional DNA-binding domain [25].

To identify a putative PRRXL1 dimerization domain, co-immunoprecipitation studies were performed with ND7/23 cell extracts co-expressing full-length PRRXL1-FLAG and the different truncated forms of HA-PRRXL1. As visible in figure 4d, PRRXL1 immunoprecipitated with itself and with $PRRXL1_{1-227}$, $PRRXL1_{1-180}$ and $PRRXL1_{107-263}$ truncated forms. This implies that these three truncations have a sequence capable of binding PRRXL1. However, $PRRXL1_{1-143}$ was not capable of binding, together with $PRRXL1_{189-263}$, which are constructs lacking aminoacids 143-180. This indicates the 143-180 region as the likely site for PRRXL1 homodimerization, supporting the possibility that $PRRXL1_{107-263}$ partial nuclear translocation is due to dimerization with endogenous PRRXL1.

Analysis by 2D-electrophoresis of ND7/23 cells overexpressing different PRRXL1 truncations revealed a similar 3-spot pattern for $PRRXL1_{1-227}$, $PRRXL1_{1-180}$ and $PRRXL1_{1-143}$ truncated forms (fig. 5a). On the other hand, PRRXL1 C-terminal constructs ($PRRXL1_{107-263}$ and $PRRXL1_{189-263}$) displayed a highly phosphorylated pattern, containing 5 distinct spots. These phosphorylations occurred in the cytoplasm, since the $PRRXL1_{189-263}$ truncated protein was not targeted to the nucleus (fig. 4b/c). These findings suggest that there are at least two phospho-sites between aminoacids 1-143 and four phospho-sites from aminoacids 189 onwards. In fact, as $PRRXL1_{1-227}$ truncation displayed a similar spot pattern as the other N-terminal constructs, the majority of the C-terminal phosphorylations must occur from aminoacid 227 forward.

To further study this PRRXL1 C-terminal phosphorylation, a chemical cleavage assay with CNBr, which cuts at methionine residues (fig. 5b), was performed. To identify the cleaved fragments, ND7/23 cells overexpressing PRRXL1-HA fusion protein were shortly incubated (20 minutes) with CNBr (fig. 5b) and compared with the migration of $PRRXL1_{107-263}$ and $PRRXL1_{189-263}$ constructs. The small amount of the $PRRXL1_{69-263}$ on WB suggests that this is a fragment more prone to cleavage in the first methionine residues, thus originating high level of both $PRRXL1_{107-263}$ and $PRRXL1_{189-263}$. This observation correlates well with surface accessibility scores, according to which PRRXL1 N-terminal region containing the homeodomain is highly exposed [26,23]. In 2D electrophoretic analysis of PRRXL1-HA treated with prolonged CNBr incubation (fig. 5c), a new fragment encompassing aminoacids 212 forward was detected. It still displayed the same number of phosphorylated spots (four) as the $PRRXL1_{189-263}$ peptide, confirming that the majority of C-terminal phosphorylations occur from aminoacid 212 forward.

One advantage of the CNBr chemical cleavage, in comparison with the truncation approach, is the use of full-length PRRXL1, which maintains its native conformation. Thus, contrary to the $PRRXL1_{189-263}$ truncation (fig. 5a), the spot pattern of $PRRXL1_{189-263}$ resulting from the CNBr cleavage was also vertically resolved, while the highly phosphorylated $PRRXL1_{212-263}$ fragment was just horizontally resolved (fig. 5c). This indicates that this part of the protein may not suffer SDS-PAGE resistant conformational changes. Therefore, the data suggest that the structural alterations occurring in PRRXL1 must include the region up to aminoacid 212, and comprises most of the *otp, aristaless and rax* (OAR) domain. However, these structural changes were not visible in the $PRRXL1_{189-263}$ truncation, which was never expressed in the presence of the N-terminus of the protein. Therefore, this may indicate that the N-terminal region is necessary for the induction of the mentioned structural changes. As the $PRRXL1_{107-263}$ truncation was also vertically resolved, the region important for PRRXL1 conformational changes to occur must be located between aminoacids 107 and 189. This can be further narrowed to region 107-143, as $PRRXL1_{1-143}$ was the smallest N-terminus truncation that

exhibited a conformation change. Overall, two phosphorylation clusters were identified, one encompassing aminoacids 1-143 and containing at least 2 phosphorylated residues, and other encompassing aminoacids 227-263, containing at least 4 phosphorylated residues. These regions likely have structural implications for the phosphorylation rich C-terminus.

PRRXL1 C-terminal region contains a neuronal-specific repressing domain

Protein phosphorylation has been described as an activity regulator of several transcription factors [27], which could also be the case for PRRXL1. RGMb is a protein whose expression is known to be regulated by PRRXL1 [15], and thus a luciferase reporter assay was used to measure PRRXL1 activity on the *Rgmb* promoter in ND7/23 and non-neuronal HeLa cell lines (fig. 6a). Comparison of PRRXL1 transcriptional activity with the control (empty vector) revealed a repressor function for PRRXL1 in both models. However, deletion of PRRXL1 C-terminus (aminoacids 227 to 263) diminished PRRXL1 repressor activity in ND7/23, but not in HeLa cells, which is suggestive of a neuronal-specific effect. As expected, $PRRXL1_{107-263}$ and $PRRXL1_{189-263}$ did not change basal promoter activity, since they lack the ability to bind DNA.

When looking at DNA affinity (fig. 6b), a great reduction in DNA binding was observed in constructs with deletion of the homeodomain, as expected. Conversely, constructs lacking the C-terminus regulatory domain still maintained their ability to bind DNA (fig. 6b, $PRRXL1_{1-143}$). However, full-length PRRXL1 expressed in *E. coli*, where PRRXL1 is not phosphorylated or folded (fig. 1a), was also not able to bind DNA. This reinforces the role of phosphorylation on PRRXL1-DNA binding, and thus PRRXL1 activity as a transcription factor.

DISCUSSION

PRRXL1 is a paired-like homeodomain transcription factor with a crucial role in the differentiation, connection and maintenance of DRG and dSC pain-processing neurons during development [6]. However, the mechanisms governing PRRXL1 activity in nociceptive system development are unknown. This study shows that PRRXL1 is phosphorylated. These phosphorylated residues are located in two well defined functional domains: one encompassing aminoacids 1-143 and involved in conformational changes, and the other including aminoacid 227 forward and displaying neuronal specific repressor activity (fig. 7).

Protein phosphorylation is the most common post-translational modification in eukaryotes [9]. It is frequently used in signal transduction, and it is implicated in basic cellular processes such as metabolism, growth, division and differentiation [28]. In fact, several homeodomain proteins have been reported to be phosphorylated, such as Arix [27], Csx/Nkx2.5 [12] and more recently Pax6 [29]. PRRXL1 acquires distinct phosphorylation patterns along SC and DRG development, with higher phosphorylation states at early developmental ages (from E10.5 to E14.5). At later stages, PRRXL1 is progressively less phosphorylated, thus acquiring a more compact and stable protein conformation with reduced transcriptional activity. Therefore, reversible phosphorylation and dephosphorylation can act as a molecular switch on PRRXL1 function, with important impact on regulating diverse cellular functions, as occurs with several other proteins [30,31].

However, this change in phosphorylation state does not occur at similar time points in these tissues. PRRXL1 is maintained at a hyperphosphorylated state until later ages (P7) in the DRG when compared to the SC (E18.5). Moreover, while in the SC there is a progressive

conversion of bands 1/2 to band 4, PRRXL1 in the DRG progressively acquires a conformation corresponding to band 3 even at late post-natal ages. Thus, one may argue that specific phospho-sites are spatially and chronologically regulated. Considering that the anatomo-functional abnormalities in *Prrxl1*$^{-/-}$ mouse occur earlier in the dSC than in the DRG [6,32], this indicates that PRRXL1 might play different roles in different tissues, supporting the proposed PRRXL1 involvement in neuronal differentiation within the dSC, and in the DRG-spinal targeting within the DRG [32]. Mechanistically, this probably results from distinct phosphorylation states acquired by PRRXL1 during the different phases of DRG and SC neurodevelopment, which may promote the association of PRRXL1 with distinct molecular partners/promoter regions. The combinatorial interaction between various homeodomain transcription factors indeed commands the differentiation of SC and DRG neuronal subpopulations [3]. Differential expression of PRRXL1 with LMX1b and TLX3 in the dSC defined three different *laminae* I-III glutamatergic neuronal populations [32]. PRRXL1 could therefore be involved in this mechanism through phosphorylation.

PRRXL1 has at least six phosphorylated residues, as determined by the 2D electrophoretic analysis of mouse SC and DRG tissue. These phosphorylations can be grouped in two clusters. Therefore, there are at least two phospho-sites between aminoacids 1-143 and four phospho-sites in the C-terminus (aminoacids 227-263). The most common phosphorylated aminoacid residues are phosphoserine and phosphothreonine [9], which is probably the case for PRRXL1. In fact, phosphoserine-binding domains have been described to play an important role in signal propagation, by enabling phosphorylation-dependent protein-protein interactions [11]. If this is the case for PRRXL1, such phosphorylated residues could play a role in PRRXL1 signaling pathway. Additionally, protein kinases target many cellular proteins and can cause significant conformational changes. On the other hand, protein conformation is very important for kinase accessibility, and therefore for protein activity. Therefore, PRRXL1 conformational changes must act as a mechanism to regulate the interaction between PRRXL1 and other proteins involved in the development of the nociceptive system. Phosphorylation seems to induce a less stable conformational change, and CNBr cleavage assays suggest that the N-terminal is highly exposed, a condition important for PRRXL1-DNA binding. The structural alterations occurring in PRRXL1 result in a four band pattern upon SDS-PAGE. This four band pattern results from two similar paired bands (bands 2/4 and the not so clearly visualized bands 1/3) that likely result from phosphorylation at key residues. According to this work, some of these residues must be located in the region of PRRXL1 encompassing aminoacid residues 107-143.

It is known that PRRXL1 has an homeodomain and an OAR domain [33]. The homeodomain is a 60 aminoacid region that binds to palindromic DNA regulator sequences [34]. Here, a phosphorylation cluster encompassing this homeodomain was identified. Considering this proximity, it is hypothesized that this region may regulate PRRXL1-DNA binding events. This is further supported by the fact that PRRXL1 expressed in a prokaryotic system, and thus not phosphorylated, is not able to bind DNA. On the other hand, the OAR domain spans aminoacids 199-219 and is a transactivation domain whose function is not yet fully understood. However, *in vivo* and *in vitro* studies have shown that the deletion of the OAR domain in the Prx1/2 and Cart1 homeodomain proteins increases DNA binding and transcriptional activity [35-37]. Thereby, it has been proposed that the OAR domain negatively modulates the protein function, which may in this case be aided by the phosphorylation of PRRXL1 identified repressor domain. This negative regulation of transcriptional activity may also involve an intra-molecular interaction between the OAR and the DNA-binding homeodomain [35]. Since these two functional domains are located at opposite ends of the

PRRXL1 protein, an interaction between them will most likely require a structural loop, mediated through the phosphorylation of the conformation domain.

Recently, the existence of a "post-translational modification code" has been proposed, in a way that the distinct combination of post-translational modifications could function as a molecular code, thus originating a large set of combinations from a limited set of elements and conveying different meanings or biological responses [38]. The existence of such a code could be extrapolated to a "phosphorylation code", in that different combinations of phosphorylated residues would convey different molecular responses. In fact, this has already been described for the retinoblastoma tumor suppressor protein (Rb) [39]. It is known that different phosphorylated forms of Rb exist, and it was demonstrated that phosphorylation events induce unique conformations in Rb. These conformational changes are site specific and provide a mechanism through which different phosphorylation events can code for different functional Rb outputs [39]. This could also be true for PRRXL1, in a way that different combinations of phosphorylated residues could enable a wider and at the same time a more specific range of action. The existence of phosphorylated residues within a given PRRXL1 domain could further increase its transcriptional potential. Therefore, a putative PRRXL1 phosphorylation code could enable fine-tuning of transcriptional responses beyond a mere global modulation of transcription factor concentration, further augmenting PRRXL1 capabilities in the regulation of nociceptive system development.

In conclusion, the present findings give important insight on phospho-dependent functioning of PRRXL1, and uncover a part of the vast mechanisms that take part in regulating PRRXL1 activity. However, the exact phosphorylated aminoacid residues will be invaluable for further study of the role of PRRXL1 in the development of the nociceptive system, and the ND7/23 cell line appears to be a suitable model for further studies on PRRXL1 transcriptional activity.

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FIGURES, TABLE AND LEGENDS

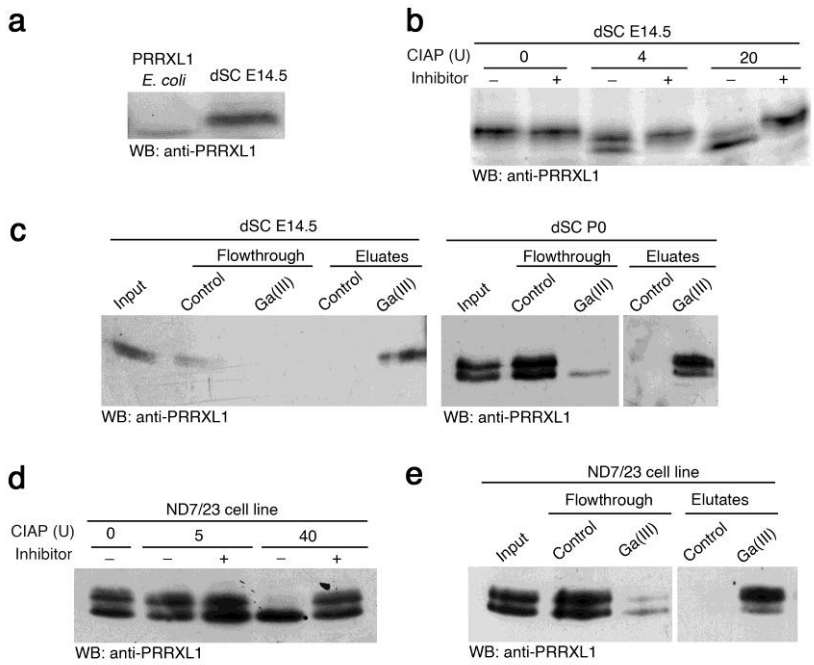

Fig. 1 – PRRXL1 multiple band pattern is the result of phosphorylation. **a)** Comparison of PRRXL1 expression pattern in *Escherichia coli* and in dSC from E14.5 and P0 mice. dSC extracts migrate as a multiple band pattern, while PRRXL1 expressed in *E. coli* migrates as a lower single band. **b)** PRRXL1 band pattern is a result of phosphorylation. Lysates from E14.5 dSC were treated with different units (U) of CIAP in the presence (+) or absence (-) of a competitive inhibitor (Na_2HPO_4), resulting in progressive elimination of the multiple band pattern. **c)** Phosphoprotein enrichment by Ga(III) IMAC of protein lysates from E14.5 and P0 dSC. The upper bands in all samples are enriched in the eluate. The control matrix (without Ga^{3+}) did not bind PRRXL1. **d)** ND7/23 cell overexpressing *Prrxl1* were incubated with CIAP, resulting in the abrogation of the multiple band pattern. **e)** Ga(III) IMAC of protein lysates from ND7/23 cells overexpressing PPRXL1 shows upper band enrichment.

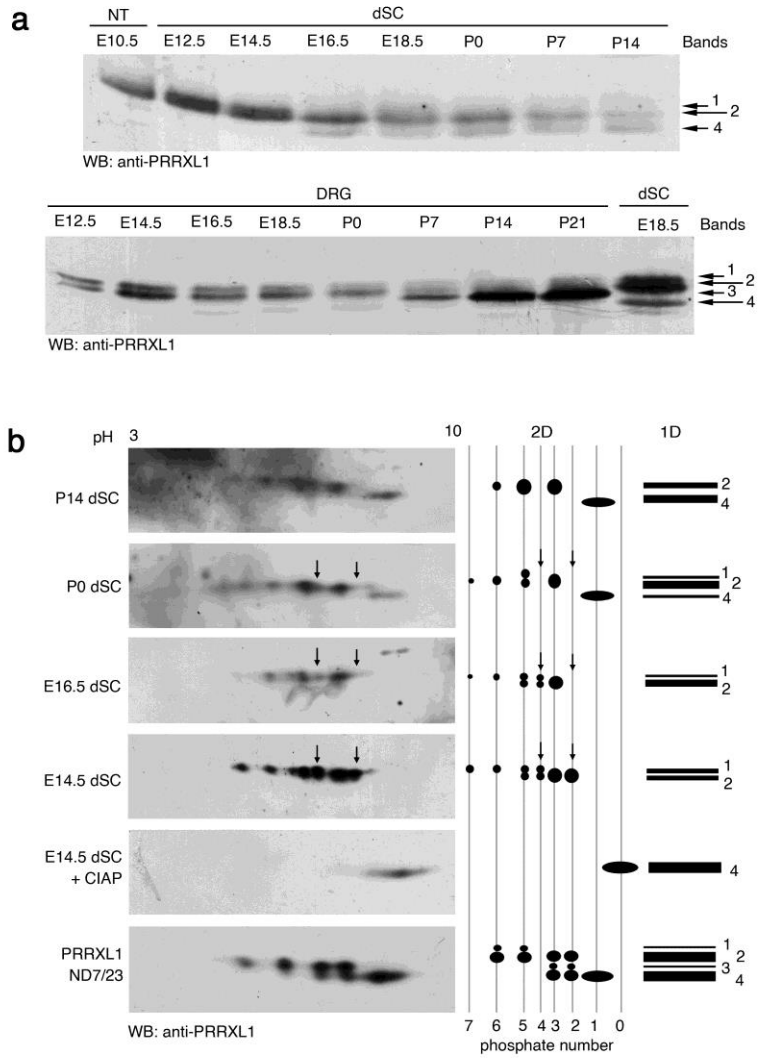

Fig. 2 – PRRXL1 acquires distinct phosphorylation profiles along development. **a)** WB characterization of PRRXL1 band pattern at distinct dSC and DRG developmental ages. Loaded protein extracts were equilibrated to the overall PRRXL1 signal in order to highlight differences in the band pattern. As development progresses, the lower bands grow in relative abundance. Note that the lowest band in the DRG tissue is band number 3 (compare P21 DRG and E18.5 dSC). **b)** 2D analyses of PRRXL1 from dSC (E14.5, E16.5, P0 and P14) and ND7/23 cells overexpressing PPRXL1. E14.5 dSC treated with CIAP was used to identify the PRRXL1 dephosphorylated state. For each sample, a correspondence between the 2D differential spot pattern with the 1D SDS-PAGE band pattern was depicted in the diagram. The arrows indicate variation of particular spots along development. The number of phosphorylated residues was estimated by using the theoretical p*I* of multisite phosphorylated Prrxl1 and the dephosphorylated control (CIAP). *NT: neural tube.*

Table 1 - Ferguson plot analysis of PRRXL1 upper doublet *vs* lower doublet.

	Mean Kr	S.E.M.	Y_0	S.E.M.
PRRXL1 (bands 1/2)	-4.710	0.260	2.151	0.264
PRRXL1 (bands 3/4)	-4.830	0.269	2.308	0.282
Average difference	0.121	0.010	0.156	0.022
p	0.0062		0.008	

Table 1 – Ferguson plot analyses correlates PRRXL1 phosphorylation-induced gel shift with distinct protein shapes. A linear regression of the logarithm of the relative electrophoretic mobility as a function of acrylamide concentration was performed. The table shows the slope (which corresponds to the retardation coefficient (Kr)) and the y-intercept (y_0). Differences in Kr relate to differences in molecular radius while differences in y_0 are related to differences in charge. This shows PRRXL1 phosphorylation induces a change in the molecular radius of PRRXL1. *Kr: slope of electrophoretic mobility. S.E.M.: standard error of the mean. Y_0: y-axis intercept.*

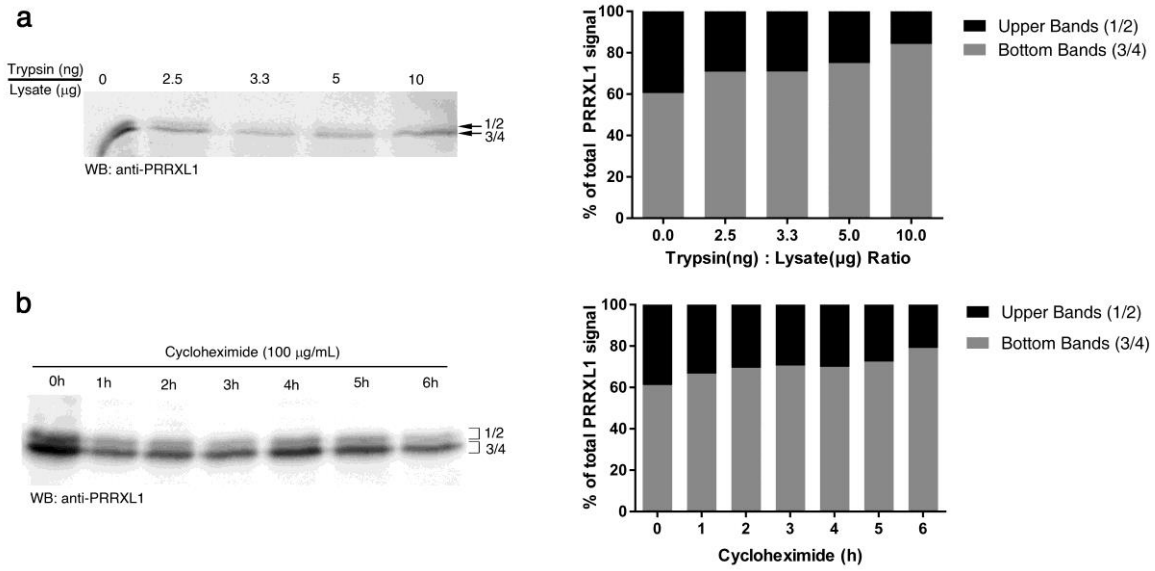

Fig. 3 - PRRXL1 suffers phospho-dependent conformational changes. **a)** Limited tryptic proteolysis. ND7/23 extracts overexpressing *Prrxl1* were treated with different amounts of trypsin and analyzed by WB. The graph represents the signal quantification of PRRXL1 upper (1/2) *versus* lower (3/4) bands. PRRXL1 upper bands are more prone to digestion, suggesting a more exposed conformation. **b)** PRRXL1 protein stability. ND7/23 cells overexpressing PRRXL1 were treated with cycloheximide and the PRRXL1 band abundance was assessed overtime by WB. The graph represents the signal quantification of PRRXL1 upper (1/2) *versus* lower (3/4) bands. The results are representative of three independent experiments.

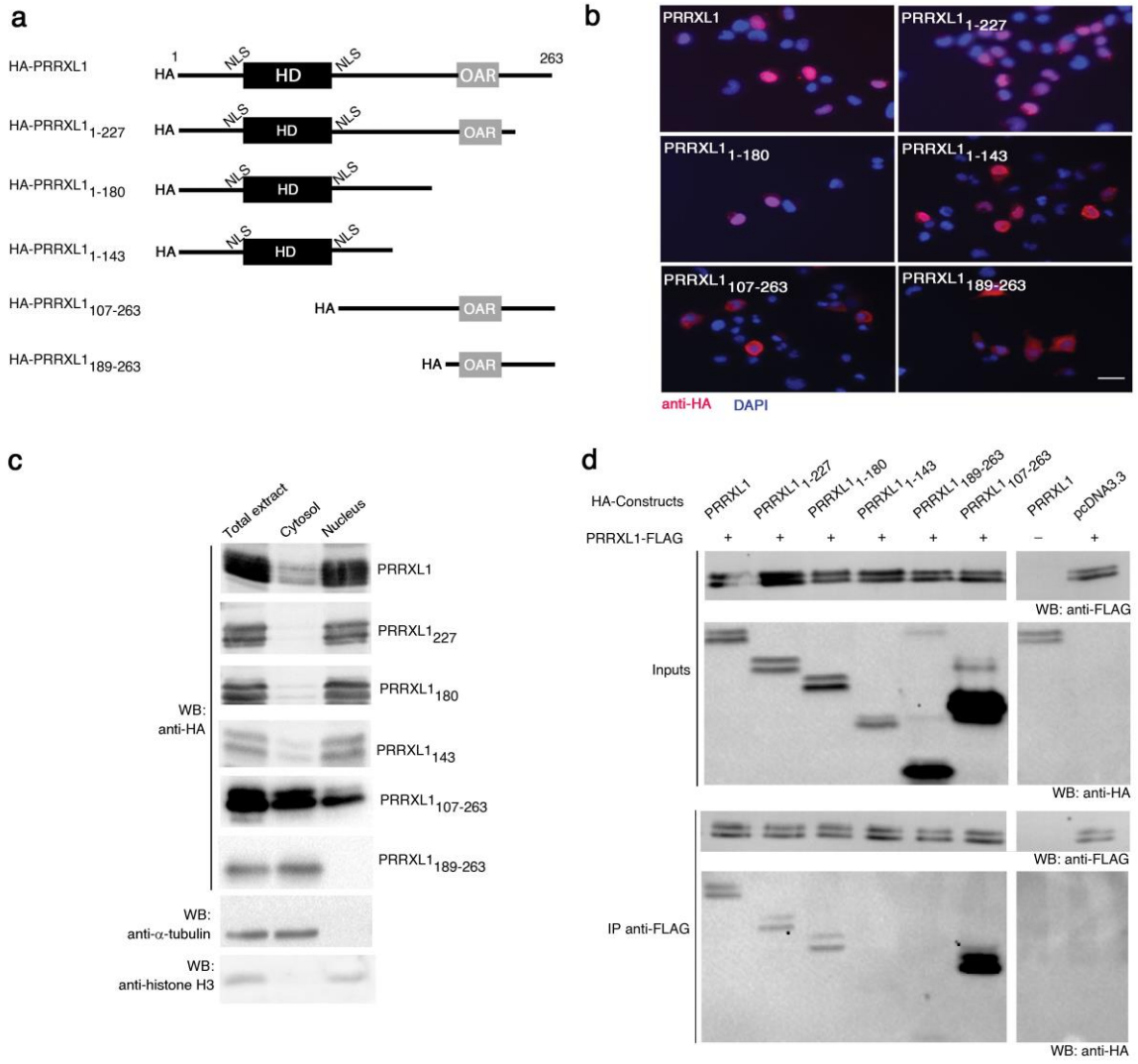

Fig. 4 – Analysis of PRRXL1 truncated forms. **a)** Schematic representation of HA-PRRXL1 truncated forms. **b)** Immunocitochemistry of truncated versions of HA-PPRXL1. Anti-HA antibody stains the truncated forms in red, and DAPI stains the cellular nuclei in blue. Co-localization of the two stains suggests nuclear translocation of the analyzed truncations. **c)** Subcellular fractionation of PRRXL1 constructs. The cytosolic and nuclear fraction were validated by using anti-tubulin and anti-histone H3 antibody respectively. **d)** Identification of PRRXL1 dimerization domain. Co-immunoprecipitation studies with full-length PRRXL1-FLAG and HA-PRRXL1 truncated forms reveal aminoacids 143-180 as the likely site for PRRXL1 homodimerization.

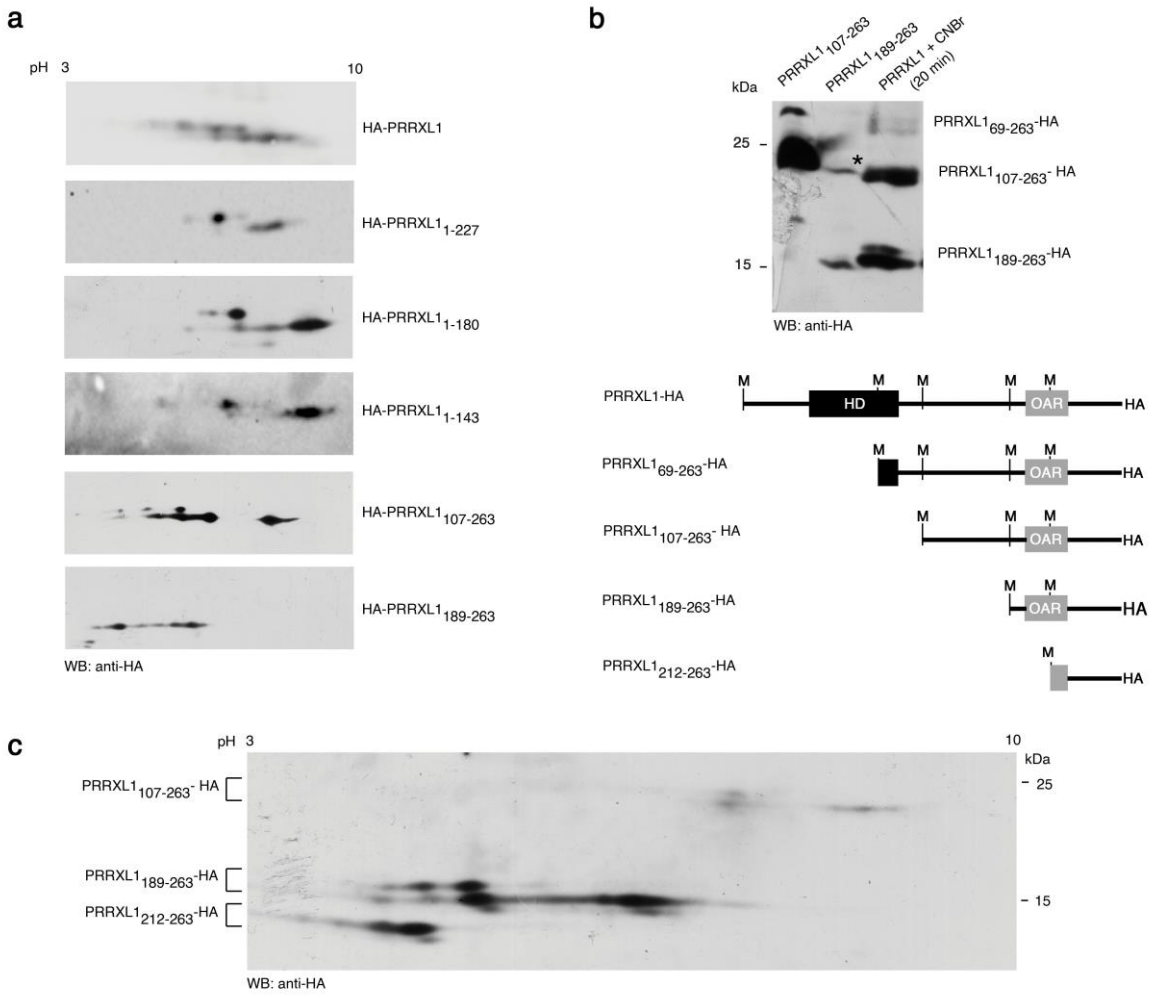

Fig. 5 – Mapping of PRRXL1 phosphorylated regions. **a)** 2D electrophoresis of ND7/23 extracts overexpressing PRRXL1 truncated forms. **b)** Short CNBr cleavage assays (at methionine residues) were performed using overexpressed PRRXL1-HA and analyzed by WB with anti-HA antibody. The scheme represents theoretical PRRXL1 fragments, which were validated by comparison with the apparent size of the $PRRXL1_{107-263}$ and $PRRXL1_{189-263}$ constructs. The asterisk indicates a dimeric form of $PRRXL1_{189-263}$. **c)** 2D electrophoretic analysis of ND7/23 cell extracts overexpressing PRRXL1-HA previously subjected to prolonged CNBr cleavage, revealing $PRRXL1_{212-263}$.

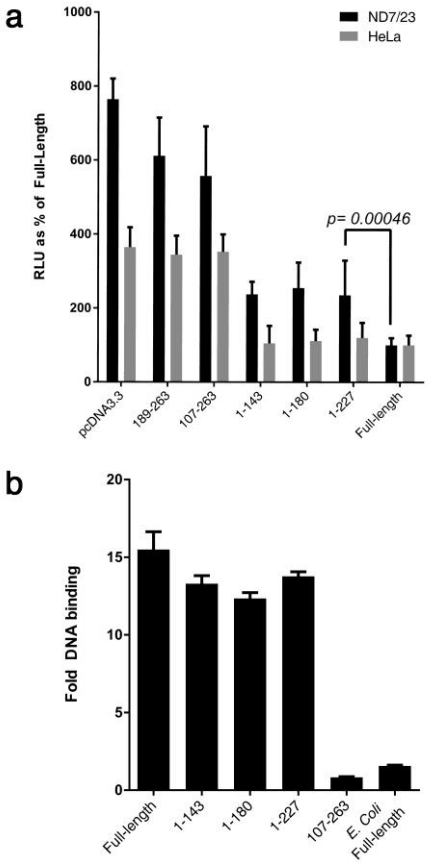

Fig. 6 – PRRXL1 C-terminal contains a neuronal-specific repressor. **a)** Transcriptional activity of PRRXL1 truncations. Luciferase assays on *Rgmb* promoter were performed with ND7/23 and HeLa cells extracts expressing different PRRXL1 constructs. **b)** DNA-binding analysis of PRRXL1 truncations. ND7/23 cell extracts expressing different PRRXL1 truncations and recombinant PRRXL1 purified from *E. coli* were processed for DNA pull-down plate assays in the presence or absence (control) of a biotinylated *Rgmb* promoter probe. Values are represented as fold enrichment relative to the control sample.

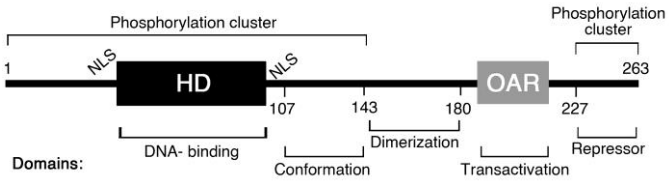

Fig. 7 – Schematic representation of PRRXL1 functional domains and the location of putative phosphorylated regions.

Supplementary Table 1

Construct	Lower (Forward)	Upper (reverse)
PRRXL1 (for pGEX-4T3 cloning)	CCGGAATTCCATGTTTTATTTCCACTGTCCGCCAC	ATAGTTTAGCGGCCGCTCATACACTCTTCTCTCCCTC
PRRXL1-HA	GGAATTCGCCACCATGTTTTATTTCCACTGTCCGCCAC	TCAAGCGTAATCTGGAACATCGTATGGGTATACACTCTTCTCTC
PRRXL1-FLAG		TCATTTATCGTCATCGTCTTTGTAGTCTGCGGCCTGTACACTCTTCTCTC
PRRXL1		GCTCTAGATCATACACTCTTCTCTCCC
HA-PRRXL1	CGCCACCATGTACCCATACGATGTTCCAGATTACGCTTTTTATTTCCACTGTCCGCC	
HA - PRRXL1$_{1-143}$		TCACACTGTGCGTCCCAGGCT
HA - PRRXL1$_{1-180}$		GCACAGTGGGCCCCCTTT
HA - PRRXL1$_{1-227}$		CAGGAGGTTGGCAGACTG
HA - PRRXL1$_{107-263}$	CGCCACCATGTACCCATACGATGTTCCAGATTACGCTGCAGAGGTGACACCACCG	GCTCTAGATCATACACTCTTCTCTCCC
HA - PRRXL1$_{189-263}$	CGCCACCATGGGACTCTCCTTCCTC	

Supplementary Table 1. Primers used for cloning all studied constructs.

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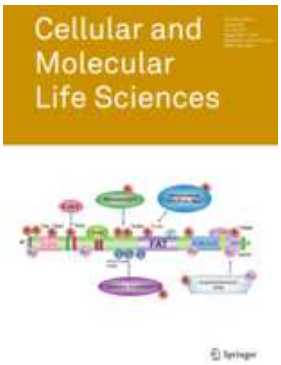

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  Reviews should be overviews of recent developments in research fields of general interest, with a fair representation of different aspects of the topic. The format,

including introduction, headings, subheadings, and discussion, will vary depending on the subject. Reviews should not contain unpublished original results of the author.

CMLS does not have obligatory length restrictions. However, as a guideline, the length of a review article should not exceed 20 typeset pages, including figures, tables and references (a printed page contains approx. 1000 words or 40 references).

- Letters and Comments

  In addition to manuscripts, letters or comments discussing an article published in the past 6 months in CMLS are welcome and may appear in print in one of the following issues.

  Each comment will be sent to the corresponding author of the discussed article together with an invitation to respond. The comment and the reply may be sent to an independent referee. However, the editor-in-chief will make the final decision whether the correspondence will be published. CMLS allows the original author a deadline of two weeks to reply. If no answer is received within this timeframe, the editor-in-chief will decide if the letter or comment will be published without response. Letters and comments may be edited and shortened for reasons of clarity and space.

  In order to avoid long disputes, only the original comment together with the author's response will be published. Any additional comments or remarks should be sent to the authors directly and will not further be handled by CMLS.

  Letters and comments should not exceed 2-3 typed, double spaced pages with only a few essential references, if necessary. Figures should be avoided, unless there is a special need to clarify the written content. A separate page should include a short title, the writer's name and affiliations, as well as the exact citation of the article commented on.

- Special Sections

  The special sections "Visions and Reflections" and "Memories of a Senior Scientist" provide a forum for discussing expert opinions, considerations and suggestions by leading scientists who have made important contributions and whose advice may be inspiring and educational to the young scientific generation.

  Contributions in these sections must refer to a subject within the scope of CMLS (biochemistry, cell biology, molecular genetics, molecular and cellular aspects of biomedicine, immunology, neuroscience, pharmacology and physiology).

- "Visions and Reflections"

  This type of article provides a forum for expert opinions on recent developments in research fields of general interest, including the opportunity of informed speculation on present and future developments.

  "Visions and reflections" articles are usually solicited by a personal invitation. However, unsolicited manuscripts are considered as well. In that case the editorial staff will decide on the general suitability of the paper for CMLS. Manuscripts found unsuitable will be returned to the authors within less than a week. All manuscripts are subject to peer review by at least two external reviewers.

  CMLS does not have obligatory length restrictions. However, as a guideline, the length of a Visions/Reflections article should not exceed 8 typeset pages, including figures, tables and references (a printed page contains approx. 1000 words or 40 references).

- "Memories of a Senior Scientist"

  These contributions may be either a brief scientific autobiography or, preferably,

refer to a particular scientific highlight to which the author has contributed in some way. Subjects should be selected for their inspirational or educational value for the young scientific generation.

Contributions are usually invited by the editor-in-chief but unsolicited manuscripts may also be considered. In that case, the editorial staff will decide on the general suitability of the paper for CMLS. Manuscripts found unsuitable will be returned to the authors within less than a week. Final acceptance of all manuscripts in this section is subject to the discretion of the editor-in-chief.

CMLS does not have obligatory length restrictions. However, as a guideline, the length of a Memories article should not exceed 8 typeset pages, including figures, tables and references (a printed page contains approx. 1000 words or 40 references). A picture of the scientist should be joined and will be reproduced.

EDITORIAL PROCEDURE

All manuscripts received are acknowledged immediately. All submitted manuscripts, including invited articles, are subjected to a two-tier evaluation process. In a first step the editorial staff decides on the general suitability of the paper for CMLS. Manuscripts found unsuitable will be returned to the authors within less than a week. All other manuscripts are subjected to peer review involving at least two external reviewers. Authors will be notified of the editorial decision based on the recommendations of the reviewers (in general within one month after submission).

Manuscripts must be accompanied by a covering letter, which should include a brief explanation of the importance of the subject and how it fits within the scope of CMLS.

Authors are requested to provide the names and full addresses (including e-mail address) of up to four potential referees, with whom they have not worked before. Persons to be excluded from refereeing may be named as well.

GUEST EDITORS OF MULTI-AUTHOR REVIEWS

- One of the unique features of Cellular and Molecular Life Sciences (CMLS) is the publication of 'multi-author reviews' (MARs), a collection of usually 6-12 review articles. Together they comprehensively cover a defined scientific field of outstanding interest. The range of topics covered by MAR corresponds to the overall scope of CMLS (see above).
- A MAR is organised by a Guest-Editor upon invitation by the editor-in-chief. Successful completion of a MAR is a long term endeavour and requires much energy and expertise of the guest-editor. Accepting the invitation to guest-edit a MAR means engaging in a long term commitment and responsibility.

The consecutive tasks of the guest editor entail:

• Establishing a preliminary list of potential authors and topics covering the different aspects of the field. Submitting the list to the editor-in-chief for approval. The list should be a fair representation of the subject, it should not be dominated by contributions of a single laboratory or a single school of thought.

• Invitation of authors following approval and submitting a final list of authors (including titles, affiliations, contact addresses, etc) who have agreed to contribute. Coordinating their contributions, including organization of contacts between authors to avoid overlap and streamline mutual complementarity. The editorial office will also contact the authors and send out instructions for authors.

• Writing an introduction. In addition, the guest-editor may contribute one of the reviews or an

optional summary. Introduction and summary should be brief and concise.

• Critically reviewing and editing the collected manuscripts, including references and figures. Asking the authors for revisions, if necessary. Technical terms should be checked by the guest editor and should be as uniform as possible throughout all articles of the review.

• Prompting authors to adhere to deadlines.

• Submitting the completed MAR to the editorial office for final approval by the editor-in-chief.

The Publisher is responsible for proofreading and for language editing of manuscripts.

A deadline for submission is set between the guest-editor and editor-in-chief by mutual agreement. The guest editor should insist that the authors deliver their manuscripts to him at least 3 months before the anticipated submission date to allow sufficient time for reviewing, revisions and editing. Publication will be 2-3 months after complete submission of the MAR. The editorial office will try as much as possible to relieve the guest editor of routine administrative work.

The individual reviews of a MAR (no less than 5) should be prepared according to the instructions for ordinary reviews (see above), except that the recommended length should not exceed 16 typeset pages, including figures, figure legends, tables and references (a printed page contains approx. 1000 words or 40 references).

Review Articles as Part of a Multi-Author Review

The individual reviews of a MAR should be prepared according to the instructions for ordinary reviews, except that the recommended length should not exceed 16 typeset pages, including figures, figure legends, tables and references (a printed page contains approx. 1000 words or 40 references).

Authors will be invited by the guest-editor and will submit their articles to him. The guest-editor of the multi-author review takes care of the reviewing of the individual review articles, requests revisions if necessary, and decides about acceptance. As soon as the editorial office of CMLS receives the accepted reviews from the guest editor they will be acknowledged immediately.

MANUSCRIPT SUBMISSION

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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Authors should submit their manuscripts online. Electronic submission substantially reduces the editorial processing and reviewing times and shortens overall publication times. Please follow the hyperlink “Submit online” on the right and upload all of your manuscript files following the instructions given on the screen.

TITLE PAGE

Title Page

The title page should include:

- The name(s) of the author(s)
- A concise and informative title
- The affiliation(s) and address(es) of the author(s)
- The e-mail address, telephone and fax numbers of the corresponding author

Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

TEXT

Text Formatting

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX.

LaTeX macro package (zip, 182 kB)

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section before the reference list. The names of funding organizations should be written in full.

REFERENCES

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].
2. This result was later contradicted by Becker and Seligman [5].
3. This effect has been widely studied [1-3, 7].

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

The entries in the list should be numbered consecutively.

- Journal article

  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8

  Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI

  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

  Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal’s name according to the ISSN List of Title Word Abbreviations, see

www.issn.org/2-22661-LTWA-online.php

For authors using EndNote, Springer provides an output style that supports the formatting of

in-text citations and reference list.

EndNote style (zip, 2 kB)

Authors preparing their manuscript in LaTeX can use the bibtex file spbasic.bst which is included in Springer's LaTeX macro package.

Please use the output style for European Journal of Applied Physiology when using EndNote reference manager.

TABLES

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

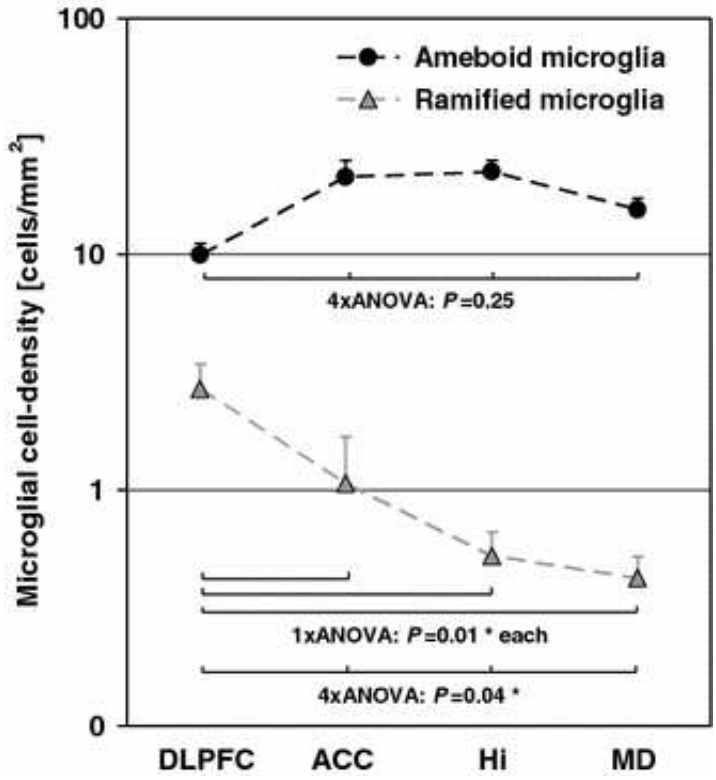
ARTWORK AND ILLUSTRATIONS GUIDELINES

For the best quality final product, it is highly recommended that you submit all of your artwork – photographs, line drawings, etc. – in an electronic format. Your art will then be produced to the highest standards with the greatest accuracy to detail. The published work will directly reflect the quality of the artwork provided.

Electronic Figure Submission

- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MS Office files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

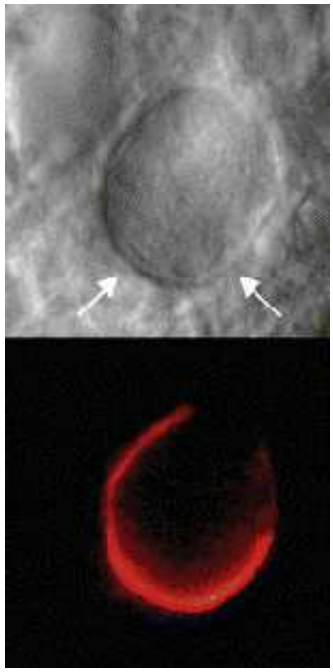
Line Art



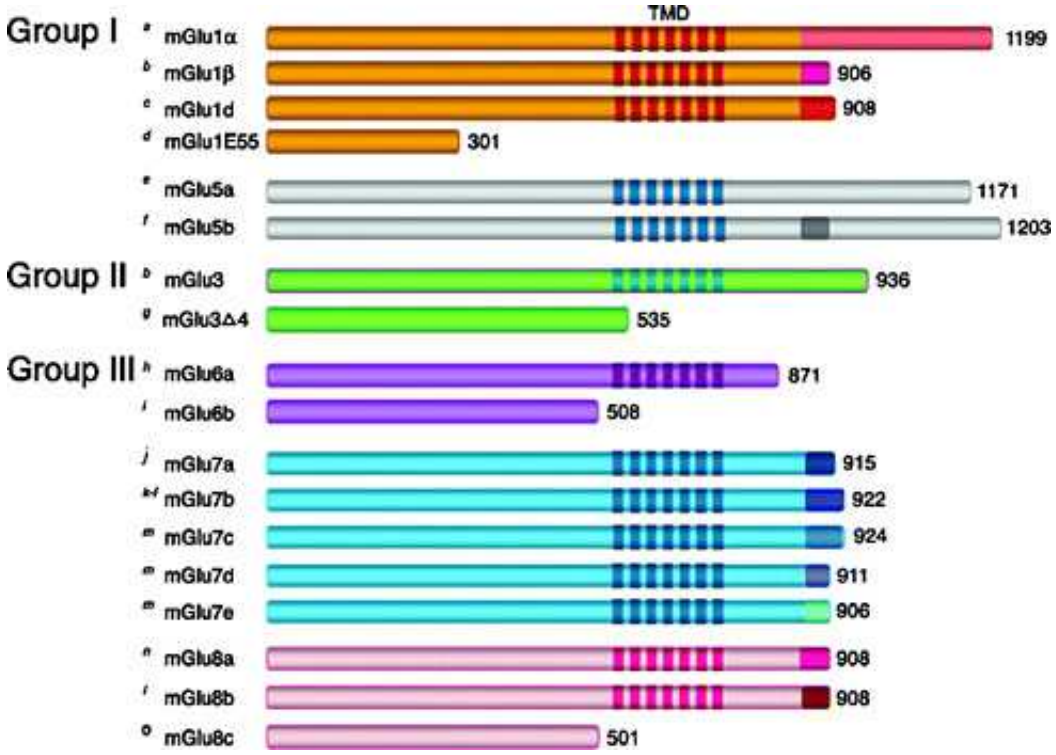
- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.
- Vector graphics containing fonts must have the fonts embedded in the files.

Halftone Art

- Definition: Photographs, drawings, or paintings with fine shading, etc.
- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.
- Halftones should have a minimum resolution of 300 dpi.



Combination Art



- Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.
- Combination artwork should have a minimum resolution of 600 dpi.

Color Art

- Color art is free of charge for online publication.
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- To add lettering, it is best to use Helvetica or Arial (sans serif fonts).
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- All figures are to be numbered using Arabic numerals.
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- If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures,

"A1, A2, A3, etc." Figures in online appendices (Electronic Supplementary Material) should, however, be numbered separately.

Figure Captions

- Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.
- Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.
- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.
- Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.
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- When preparing your figures, size figures to fit in the column width.
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