

**Transcription Factor Positive Regulatory Domain 4 (PRDM4) recruits Protein Arginine Methyltransferase 5 (PRMT5) to mediate histone arginine methylation and control neural stem cell proliferation and differentiation.**

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**\*Running Title:** PRDM4 controls NSC differentiation

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**Keywords:** histone arginine methylation; neural stem cells; PRDM4; PRMT5; SC1

**Background:** Neural stem cells generate all the cell types of the central nervous system.

**Results:** Transcription factor, PRDM4, recruits protein arginine methyltransferase 5 (PRMT5) to control the timing of neurogenesis.

**Conclusions:** PRDM4 and PRMT5-mediated histone arginine methylation controls neural stem cell proliferation and differentiation.

**Significance:** Histone arginine methylation is a novel epigenetic mechanism which regulates neural stem cell reprogramming.

## SUMMARY

During development of the cerebral cortex, neural stem cells (NSCs) undergo a temporal switch from proliferative (symmetric) to neuron-generating (asymmetric) divisions. We investigated the role of Schwann cell factor 1 (SC1/PRDM4), a member of the PRDM family of transcription factors, in this critical transition. We discovered that SC1 recruits the chromatin modifier PRMT5, an arginine methyltransferase that catalyzes symmetric dimethylation of histone H4 arginine 3 (H4R3me<sub>2</sub>s), and that this modification is preferentially associated with undifferentiated cortical NSCs. Over-expressing SC1 in embryonic NSCs led to an increase in the number of Nestin-expressing precursors; mutational analysis of SC1 showed that this was dependent on recruitment of PRMT5. We found that SC1

protein levels are down-regulated at the onset of neurogenesis and that experimental knock-down of SC1 in primary NSCs triggers precocious neuronal differentiation. We propose that SC1 and PRMT5 are components of an epigenetic regulatory complex that maintains the “stem-like” cellular state of the NSC by preserving their proliferative capacity and modulating their cell cycle progression. Our findings provide evidence that histone arginine methylation regulates NSC differentiation.

## INTRODUCTION

During central nervous system (CNS) development, embryonic neural stem cells (NSCs) in the ventricular zone (VZ) of the brain and spinal cord first proliferate symmetrically to increase NSC numbers and expand the VZ, then they switch to an asymmetric mode of division to generate post-mitotic neurons while maintaining the NSC pool (1-4). After neurogenesis is complete, the NSCs switch to production of glial cells (astrocytes and oligodendrocytes). The mechanisms that control the temporal transition from proliferation to differentiation are poorly understood. Aberrations in the timing of this transition can lead to abnormalities in CNS development (5,6)

In the developing cerebral cortex NSCs generate a series of neuronal subtypes that populate different cortical layers in sequence, then switch to glial cell production (7,8). This program of neurogenesis can be recapitulated by individual mouse cortical NSCs isolated at embryonic day 10 (E10) and cultured at low density in vitro (2,3). This suggests that the temporal program of NSC division and cell fate specification is cell-intrinsic, but the molecular nature of the program is not known. The transitions in NSC fate are likely to be governed by cell lineage-specific transcription factors acting in concert with epigenetic mechanisms (9-14). The latter include post-translational modifications of histones associated with regulatory elements of genes as well as DNA methylation at CpG dinucleotides, which together affect the accessibility of chromatin to the general transcriptional machinery. The details of the epigenetic regulation of NSC differentiation are still poorly understood. In addition to cell lineage-specific transcription factors, cell cycle parameters such as the length of specific cell cycle stages play an important role in controlling NSC proliferation and differentiation (5,6,15-17) and these parameters change during cortical development (18). Lineage-specific transcription factors (5) can fine-tune the expression of cell cycle genes and in this way influence the cell fates and division modes of NSCs and consequently their decision either to proliferate or differentiate (16,19,20).

Schwann cell factor 1 (SC1) is a protein that was first identified as a binding partner of the p75 neurotrophin receptor (p75NTR) (21). SC1, also known as PRDM4, belongs to the PRDM family of proteins of which 17 members have been identified in the human genome (22). All PRDM family members are characterized by the presence of a PR (positive regulatory) domain and multiple zinc finger (ZnFg) domains. The PR domains are similar to, but distinct from, the SET domains found in many histone lysine methyltransferases (MTases) (23). PRDM proteins are either epigenetic modifiers in their own right or else they recruit third party chromatin modifiers - e.g. histone deacetylases (HDACs), histone lysine MTases or histone arginine MTases - to regulate cell type-specific gene expression in various tissues (24-33). Our previous work identified SC1/PRDM4 as an HDAC-associated transcriptional repressor that modulates cell cycle progression (33). SC1 is

highly expressed in the developing mouse cerebral cortex (34) so we set out to understand its role in the development of cortical NSCs as they switch from proliferative to neuron-generating divisions. We report that SC1 recruits a type II arginine MTase, PRMT5, that catalyzes histone H4R3 symmetric dimethylation (H4R3me<sub>2s</sub>) - a modification that we recently showed to be present in undifferentiated NSCs in the murine cortex prior to the onset of neurogenesis (35). We now show that both SC1 and PRMT5 are highly expressed in the pre-neurogenic cortex and provide evidence that the interaction between SC1 and PRMT5 regulates the proliferative capacity of cultured cortical NSCs. Our findings suggest an important role for histone arginine methylation in epigenetic programming of NSCs during cortical development.

## EXPERIMENTAL PROCEDURES

*Cell culture, transfections* - HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS) and glutamine; P19 cells were cultured in alpha-MEM (Invitrogen) supplemented with 5% FCS and glutamine; PC12 cells were cultured in DMEM supplemented with 5% FCS, 10% horse serum (HS) and glutamine. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were harvested and processed 48 hours post-transfection unless indicated otherwise.

*Immunoprecipitation, methylation assays* - HEK293T cells were transfected with described plasmids and harvested in immunoprecipitation (IP) buffer (50 mM TrisHCl pH7.4, 0.5% [v/v] NP-40, 300 mM NaCl) supplemented with a protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktails 1 and 2 (Sigma). Cells were lysed for 20 minutes on ice in IP buffer and the insoluble material sonicated for 10 s on ice. Lysates were centrifuged and the supernatants pre-cleared using protein A/G beads (Santa Cruz), then immunoprecipitated overnight at 4°C using anti-Myc (Upstate), anti-PRMT5 (Upstate) or anti-HA antibodies (Covance). The complexes were collected on protein A/G beads and washed 5 times with IP buffer, followed by a wash with cold phosphate-buffered saline (PBS) at 4°C. Proteins were

boiled at 95°C for 5 min in Laemmli buffer (60 mM Tris-HCl, pH6.8, 10% [v/v] glycerol, 2% [w/v] sodium dodecyl sulphate, 5% [v/v] β-mercaptoethanol, 0.01% [w/v] bromophenol blue) and separated by SDS-polyacrylamide gel electrophoresis (PAGE). After separation, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore) and Western blots were performed with specified antibodies in TBST buffer (100mM Tris-HCl, pH7.5, 150mM NaCl, 0.1% [v/v] Tween-20) containing 5% (w/v) skimmed milk powder (Tesco) and detected using ECL (GE Healthcare). IP's from E10.5 mouse cortices were performed using the same buffers as above. At least 20 embryos were used/IP experiment.

For methylation assays the immunoprecipitates on the beads were washed as described above and then rinsed twice in methylation buffer (50 mM Tris-HCl pH8.5, 5 mM MgCl<sub>2</sub>, 4 mM dithiothreitol [DTT], 2 μl of S-adenosyl-L-(<sup>3</sup>H)methyl methionine (<sup>3</sup>H-SAM) (Amersham, GE Healthcare) and 1 μg of histone mixture (Roche) were added to the reaction in a total volume of 30 μl. Methylation reaction was allowed to proceed for 2 hours at 30°C and stopped by adding Laemmli buffer and boiling the samples for 5 minutes. Products of the methylation reactions were separated using 15% SDS PAGE, transferred onto PVDF membranes and visualized by Coomassie brilliant blue staining and fluorography. To examine H4-specific methylation, histones were incubated with immunoprecipitated mycSC1 complex and <sup>3</sup>H-SAM as described above and analyzed on Western blots using anti-H4R3me2s antibodies (Abcam).

*Primary neural stem cell cultures* - Primary neural stem cells were isolated from mouse E10.5 cerebral cortices according to published procedures (36). Briefly, cortices were harvested in EBSS (Invitrogen) and the meninges removed. The cells were dissociated by incubation in trypsin at 37°C for 40-50 minutes. Trypsinization was stopped by adding DMEM/10% FCS. Cells were then further dissociated using pre-separation filters (Miltenyi Biotec), centrifuged gently, resuspended in a small volume of DMEM/10% FCS and plated at a density of 2.5x10<sup>5</sup> cells/13 mm poly-D-lysine coated coverslip. The cells were cultured in DMEM

supplemented with 10 ng/ml bFGF (PeproTech), 0.25% FCS, B27 supplement, Na pyruvate and glutamine (all from Invitrogen) (2). Cultures were routinely immunolabelled to monitor their ability to generate neurons, astrocytes and oligodendrocytes.

*Antibodies and immunofluorescence microscopy* - Cultured cells on coverslips were fixed in 4% (w/v) paraformaldehyde (PFA) for 10 minutes at 20-25°C and permeabilized with cold methanol for 2-3 minutes at -20°C. They were incubated for one hour at 20-25°C in blocking solution (10% normal goat serum, 0.1% [v/v] Triton X-100 in PBS). The following antibodies were used: anti-TuJ1 (Sigma, 1:500), anti-GFAP (Sigma, 1:1000), anti-Nestin (Santa Cruz, 1:400), anti-O4 (kind gift from Nigel Pringle, 1:10), anti-SC1/PRDM4 (our own antibody, 1:100, Abcam 1:100 and a gift from P Perez and MV Chao, 1:40 (33)), anti-PRMT5 (Upstate Biotech, 1:100), anti-H4R3me2s (Abcam, 1:1000), anti-EGFP (Fine Chemical Products Ltd., 1:3000), Anti-Flag (Sigma, 1:1000), anti-BrdU (American Type Culture Collection, Manassas, VA, 1:10), anti-cycB1 (GNS1, Santa Cruz Biotechnology, Inc, 1:500), anti-Pan methyl Lysine (Abcam, 1:1000). For immunolabelling with antibody O4, methanol treatment and Triton X-100 were not used. When staining for BrdU and another antigen, the cells were stained sequentially, first for an antigen of interest other than BrdU, then rinsed and treated as follows to visualise BrdU. First, the cells were fixed with 70% ethanol/ 20% glacial acetic acid mixture at RT, then in 70% ethanol at -20°C. The cells were then rinsed in PBS/1% Triton X-100 at RT and denatured in PBS/1% Triton X-100/2M HCl for 30 minutes at 37°C. After washing, anti-BrdU antibody was added overnight at 4°C. The rest of the staining was as described above. Coverslips were mounted in DAKO mounting medium (DAKO). The following secondary antibodies were used: goat anti-mouse Alexa 488, goat anti-rabbit Alexa 568, goat anti-mouse Alexa 647 (Invitrogen, 1:1000), goat anti-rat Alexa 488 (Invitrogen, 1:500). Fluorescent images were taken with a Leica Microsystems SPE confocal microscope.

E10.5 embryos were collected from timed-mated C57B/6 mice (Harlan), rinsed in PBS and fixed in 4% PFA at 4°C for 1–2 hours. Embryos were cryoprotected in 30% (w/v) sucrose in PBS and

subsequently mounted in OCT (Tissue-Tek) on dry ice. Mounted embryos were sectioned at 10 µm using a Leica cryostat and air-dried for at least 1 hour. Sections were permeabilized for 3 minutes with -20°C methanol, rinsed three times in PBS, incubated in sodium citrate buffer (10mM sodium citrate, 0.05% Tween-20, pH 6.0) at 95°C for 30 minutes for antigen retrieval, allowed to cool to room temperature, rinsed three times in PBS, then incubated for 1 hour at 20-25°C in blocking solution, following incubation with the primary antibody in blocking solution overnight at 4°C. Sections were washed three times in PBS at 20-25°C and incubated with fluorescent secondary antibodies and Hoechst dye (to visualize cell nuclei) for 1 hour at 20-25°C, rinsed three times in PBS, once in water and mounted using DAKO mounting medium. Fluorescence images were made using a Leica Microsystems SPE confocal microscope.

*siRNA Transfections* - siRNA oligonucleotides against rat SC1 were synthesized by Thermo Scientific and assessed either by applying siRNA to rat NSCs or PC12 cells and monitoring the expression of endogenous SC1 protein by Western blotting and RT-PCR or by co-transfecting SC1-specific or scrambled-sequence siRNA with an EGFP expression vector into cultured rat NSCs followed by immunolabelling with anti-EGFP and anti-SC1. We used DharmaFECT Duo Transfection reagent (Thermo Scientific) to introduce siRNA along with DNA according to the manufacturer's instructions. The cells were transfected with the siRNA oligonucleotides on two consecutive days 24 hours after plating and processed for immunocytochemistry and Western blot analysis or semi-quantitative RT-PCR 48 hours after the second application of the siRNA reagent. The following siRNA oligonucleotides were used: 1) siRNA-1 Pool: ACAAUUUGGUGCAGCUUU and GGAUGAUGUUUGUGCGCAA, 2) siRNA-2 Pool: UAAUGAUGGCCACGAAGUA and GUUCCUAUUCAGAGUCAA. Scrambled-sequence siRNAs were used as controls.

*Reverse transcription-PCR (RT-PCR)* - RNA was isolated from P19 cells or PC12 cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. 1 µg of total RNA was used for RT-PCR. Total RNA was treated with RNase-free DNase (Ambion) and cDNA

synthesized using random hexamer primers (Invitrogen) and MMLV reverse transcriptase (USB). After incubation at 42°C for 1.5 hours, the enzyme was inactivated at 75°C and the cDNA used for PCR. The following gene-specific primers were used: 1) *Rat SC1*: Fwd 5'-AAAGCCAGGAACCGTGAA-3'; Rev 5'-ATGACCCATAAAGTGAACGTG-3'; 2) *Mouse cycB*: Fwd 5'-TCCCTCTCCAAGCCCGATGG-3'; Rev 5'-TGGCCGTTACACCGACCAGC-3'; 3) *Mouse Bub1b*: Fwd 5'-AAGGGATTGAACGCAAGGCTG-3'; Rev 5'-CATCAAAAACGGTGATCCTGCG-3'; 4) *Mouse and rat GAPDH*: Fwd 5'-ACAACCTTTGGCATTGTGGAA-3'; Rev 5'-GATGCAGGGATGATGTTCTG-3'; 5) *Rat cycB*: Fwd 5'-TGGACAAGGTGCCAGTGTGCG-3'; Rev 5'-GGTCTCCTGCAGCAGCCGAAA-3'; 6) *Rat Bub1b*: Fwd 5'-GCCAGGCCCGTGGAACACAG-3'; Rev 5'-CAGGACGGAGGCACTCCCGA-3'.

## RESULTS

*Expression of SC1 in embryonic cortical NSCs* - SC1 mRNA is highly expressed in the developing cortex (34). We investigated the expression of SC1 protein in dissociated primary mouse cortical NSCs isolated at embryonic day 10.5 (E10.5) and cultured for up to 10 days in vitro (10DIV). We identified cells in these cultures by immunolabelling for Nestin (NSCs), TuJ1 (neurons), GFAP (astrocytes) or O4 (oligodendrocyte precursors, OLPs). The different cell types were generated in the appropriate temporal order (2,37): Nestin+ precursors were present from the outset, followed by TuJ1+ neurons, GFAP+ astrocytes and O4+ OLPs at progressively longer culture periods (A.C., unpublished observations). We found that immediately after plating, Nestin+ NSCs could be characterized as either strongly or weakly SC1-positive (Fig. 1A, *arrowhead* and *arrow*, respectively, quantified in Fig. 1D). Co-immunolabelling with anti-TuJ1 and anti-SC1 at 3DIV revealed that TuJ1 negative NSCs co-expressed SC1 strongly, whereas more mature neurons with high levels of TuJ1 expression, expressed low levels of SC1 (Fig. 1B). After the onset of glial differentiation at 10DIV we detected high levels of SC1 expression in all proliferating O4-positive OLPs

(Fig. 1C), but not in GFAP-expressing differentiated astrocytes (A.C., unpublished). Thus, high levels of SC1 appeared to be expressed preferentially in mitotically active cells (NSCs and OLPs) and down-regulated in differentiated neurons and astroglia. This suggested that down-regulation of SC1 might be involved in, and possibly required for, the switch from cell proliferation to differentiation.

*Knock-down of SC1 in NSCs leads to precocious neuronal differentiation* - To test whether SC1 down-regulation is sufficient to trigger NSC differentiation we examined the effects of SC1 knock-down in primary rat NSCs isolated from E11.5 cortex. We used rat NSCs in our experiment since we could knock down rat SC1 protein expression very efficiently. Transfecting two independent sets of rat SC1-specific siRNA oligonucleotides (SC1siRNA-1 and SC1siRNA-2) into cultured rat NSCs markedly decreased the expression of endogenous SC1 protein, as detected on Western blots (Fig. 2A). Moreover, SC1 immunoreactivity was low or undetectable in siRNA-transfected NSCs, compared to control siRNA-transfected NSCs (Fig. 2B). Successfully transfected cells were identified in this experiment by co-transfection of an enhanced green fluorescent protein (EGFP) expression vector. Both SC1siRNA-1 and SC1siRNA-2 gave similar results and are referred to below simply as SC1siRNA (Fig. 2 and unpublished observations). To investigate the effect of SC1 depletion on NSC differentiation, SC1-specific or scrambled-sequence siRNAs were applied twice to cultured E11.5 rat NSCs, 24 hours and 48 hours after plating. At 96 hours after plating the treated NSCs were fixed and immunolabelled with anti-EGFP to identify transfected cells and anti-TuJ1 to visualize differentiating neurons. NSCs treated with SC1siRNA consistently gave rise to ~10% of TuJ1-expressing cells with the process-bearing morphology of neurons (Fig. 2C, top panels). We did not detect a change in the number of astrocytes or oligodendrocytes after the application of SC1siRNA in these experiments within the time frame of investigation (unpublished observations). Very few TuJ1-expressing cells were observed when NSCs were transfected with control, scrambled-sequence siRNA (Fig. 2C, bottom panels). To confirm the specificity of these knock-down experiments we transfected rat NSCs with SC1siRNA together with a human SC1 cDNA

that was insensitive to inhibition by the rat-specific SC1siRNAs (Fig. 2D). This “rescued” SC1 expression in the presence of rat SC1siRNA and resulted in a reduction in numbers of TuJ1-positive neurons to control levels (i.e. as observed in NSC cultures without added siRNA or with scrambled-sequence SC1siRNA) (Fig. 2E). Consistent with the observation that a fraction of NSCs treated with SC1siRNA induced precocious neurogenesis, we found that a similar fraction of NSCs treated as above showed reduced levels of BrdU incorporation (Fig. 2F). Thus, we concluded that knock-down of SC1 in NSCs leads to precocious neuronal differentiation of a subset of the NSCs and controls their proliferative capacity, consistent with our observation that newly differentiating neurons express low levels of SC1 protein.

*SC1 is associated with histone methyltransferase activity* - Like other PRDM proteins, SC1 possesses a PR/SET domain - a hallmark of lysine histone MTases (HMTases) - which can regulate gene expression by modifying histones in chromatin (23). Therefore we tested the possibility that SC1 methylates histones as part of its transcriptional repressor function. We transiently expressed Myc-tagged SC1 (mycSC1) in HEK293T cells and immunoprecipitated mycSC1 protein from cell lysates using an anti-Myc antibody. Purified histones from calf thymus were incubated with immunoprecipitated mycSC1 and subjected to an in vitro radioactive HMTase assay (38). We detected methylated histones, the preferred substrate being histone H4 (Fig. 3A). As a positive control, we immunoprecipitated myc-Su(var)3-9, an H3K9 HMTase, and showed that this preferentially methylated histone H3 (Fig. 3A). As negative controls, we immunoprecipitated mycCREB or empty Myc vector and showed that neither of these expressed histone methylase activity (Fig. 3A). Immunoprecipitated mycSC1 also methylated recombinant histone H4 (Fig. 3B). We concluded that SC1 exhibits an HMTase activity, preferentially towards histone H4.

Histone methylation can occur on a variety of lysine and arginine residues leading to repression or activation of gene transcription, depending on the precise modification. To identify the histone modification mediated by mycSC1, the products of in vitro methylation

reactions were analyzed by Western blotting using antibodies directed against different H4 modifications. We detected an increased level of H4R3me2s in the sample containing immunoprecipitated mycSC1 (Fig. 3C, highlighted by an asterisk), while no change in overall lysine methylation was observed using an antibody directed against pan-methyl Lysine (Fig. 3C, panel 3). We concluded that mycSC1 mediates symmetric arginine dimethylation on H4R3.

*SC1 recruits the histone arginine methyltransferase PRMT5* - Given that PR/SET domains are found in *lysine* HMTases - but that we detected increased *arginine* methylation - we considered the possibility that SC1 might bind to and recruit a third-party arginine HMTase. In our methylation assays we detected an increase in H4R3me2s, a product of a type II protein arginine MTase (PRMT). Therefore, we asked whether PRMT5, a type II PRMT that is known to catalyze H4R3me2s (39,40), might interact with SC1 protein. MycSC1 expression vector was transfected into HEK293T cells and lysates were immunoprecipitated 48 hours later with anti-Myc antibody (Fig. 4A). Western blotting with anti-PRMT5 showed that endogenous PRMT5 co-immunoprecipitated with mycSC1 (Fig. 4A). HEK293T cells transfected with empty Myc vector served as a negative control. In a complementary approach, lysates of HEK293T cells transfected with mycSC1 or empty vector were immunoprecipitated with anti-PRMT5 and the precipitates analyzed on Western blots with anti-Myc. These experiments revealed that mycSC1 co-immunoprecipitated with endogenous PRMT5 (Fig. 4B).

*The amino-terminus and the PR/SET domain of SC1 are necessary to recruit PRMT5 and mediate histone methylation* - SC1 contains PR/SET and ZnFg domains characteristic of the PRDM family of proteins. To map the domain of interaction between PRMT5 and SC1 we generated a series of SC1 mutants with deletions of the PR/SET domain (mycSC1dPR), ZnFg domain (mycSC1dZF) or the NH-terminus up to the PR/SET domain (mycSC1dNH) (Fig. 4C). Full-length or truncated mycSC1 constructs were transfected together with haemagglutinin (HA)/ Flag-tagged PRMT5 into HEK293T cells and binding between pairs of expressed proteins was detected by co-immunoprecipitation assays,

using anti-HA to immunoprecipitate PRMT5 followed by Western blotting with anti-Myc to detect SC1. Co-transfection of PRMT5 and full-length mycSC1 or empty vector acted as positive and negative controls, respectively. According to this assay, mycSC1dPR bound only very weakly to PRMT5, whereas mycSC1dZF retained strong PRMT5 binding (Fig. 4D). MycSC1dNH did not bind detectably to PRMT5 (Fig. 4D). We conclude that SC1 recruits PRMT5 mainly via its NH-terminus but partly via the PR/SET domain.

Our data suggest that SC1-mediated histone arginine methylation depends on recruitment of PRMT5. To test this further we transfected mycSC1dNH (which cannot bind PRMT5) into HEK293T cells, immunoprecipitated cell lysates with anti-Myc and assayed the precipitate for HMTase activity *in vitro*. HEK293T cells transfected with full-length mycSC1 or empty vector served as positive and negative controls. We detected HMTase activity in cells transfected with full-length mycSC1 but not in cells transfected with either mycSC1dNH or empty vector (Fig. 4E). We conclude that SC1 and PRMT5 interaction is necessary for histone methylation.

*SC1 and PRMT5 are co-expressed and found in a complex in embryonic cortical NSCs* - In light of the above experiments, we asked whether PRMT5 is co-expressed with SC1 in cultured cortical NSCs. A strong PRMT5 immunofluorescence signal was detected in all Nestin-expressing NSCs isolated from E10.5 cerebral cortex (Fig. 5A). Consistent with this, we detected high levels of H4R3me2s immunoreactivity in cultured E10.5 NSCs, suggesting that there are high levels of PRMT5 enzyme activity in these cells (Fig. 5B). Moreover, we found high levels of both, PRMT5 and H4R3me2s immunoreactivity in sections through the developing cortical neuroepithelium at E10.5, as well as SC1 immunoreactivity (Fig. 5C). SC1 and PRMT5 were found in the nucleus and cytoplasm of neuroepithelial cells, suggesting that part of their mode of action might be through methylation of a cytoplasmic pool of newly synthesized histones (41). To investigate whether we can detect a complex between the endogenous SC1 and PRMT5 in the developing cortex, we performed an immunoprecipitation using mouse E10.5 cortices as the source of endogenous

PRMT5 and SC1 proteins. PRMT5 was immunoprecipitated from the cortical homogenates and the precipitates analyzed on Western blots using anti-SC1 antibodies. Endogenous SC1 co-precipitated with endogenous PRMT5, but not with a control IgG (Fig. 5D, as indicated on the figure panels, two independent co-immunoprecipitations were performed). Taken together, these data suggest that SC1 in complex with PRMT5 directs H4R3me2s modifications in proliferating cortical NSCs in vivo.

*SC1 and PRMT5 interaction is required to control the timing of NSC differentiation* - Since SC1, PRMT5 and high levels of H4R3me2s are found in the early proliferating neuroepithelium and knock-down of SC1 in NSCs leads to precocious neuronal differentiation, it seems possible that SC1 over-expression might delay the timing of neurogenesis and that the SC1/PRMT5 complex might be required for this function. To test this we transiently over-expressed either full length mycSC1 or mycSC1dNH mutant (which cannot recruit PRMT5) in cultured NSCs isolated from E10.5 mouse cerebral cortex. The cells were transfected 24 hours post-plating, fixed and double-immunolabelled 48 hours after transfection with anti-Nestin to identify the NSCs and anti-Myc to identify cells that expressed SC1. We found that over-expression of full length mycSC1 led to a moderate increase in the number of Nestin+ NSCs (Fig. 6A). This increase was not observed with mycSC1dNH, suggesting that SC1/PRMT5 interaction is necessary to control the timing of neuronal differentiation of the NSCs (Fig. 6A).

Previously, we showed that SC1 controls cellular proliferation by down-regulating pro-mitotic genes, e.g., *cycB* was one of the transcriptional targets of SC1-mediated repression (33). Therefore, we considered the possibility that the SC1/PRMT5 complex might regulate the timing of neurogenesis in developing NSCs in part by regulating their cell cycle parameters. We investigated whether SC1/PRMT5 might regulate the transcription of genes that control mitotic progression. To address this we over-expressed full-length mycSC1 and mycSC1dNH proteins in P19 embryonal carcinoma cells. P19 cells were chosen since they can be differentiated into neural lineages under defined culture conditions

(42) and are easily transfected to high efficiency. 48 hours after transfection we performed RT-PCR on mRNA isolated from P19 cells transfected with mycSC1, mycSC1dNH or Myc vector alone, and measured the expression of mitotic genes, *cycB* and *Bub1b*. We found that over-expression of full-length mycSC1, but not mycSC1dNH, led to a repression of both *cycB* and *Bub1b* (Fig. 6B). We conclude that SC1 in complex with PRMT5 down-regulates expression of certain pro-mitotic genes, e.g., *cycB* and *Bub1b*. To test whether knockdown of SC1 leads to an increase in the mRNA expression of these genes, we performed RT-PCR using total RNA isolated from PC12 cells transfected with SC1siRNA or control siRNA. PC12 cells were used as they can be transfected to a high level easily, differentiate into neurons upon treatment with Nerve growth factor (NGF) and are of rat origin allowing the use of SC1siRNA used in previous experiments with the rat NSCs. We detected an increase in the levels of *cycB* and *Bub1b* mRNA after SC1 knockdown, but not when control siRNA was used (Fig. 6C), indicating that SC1 is involved in negatively regulating the expression of these genes.

It was previously reported that a high level and activity of the cell cycle regulator *cdc2*, in association with *cycB*, is necessary for the asymmetric partitioning of cell fate determinants in neuroblasts of *Drosophila melanogaster* (20). These observations suggest that the levels and activity of pro-mitotic genes might be involved in influencing the mode of cell division adopted by the NSCs. Our observations that SC1/PRMT5 complex down-regulates the expression of *cycB* and that SC1 protein levels are down-regulated in the newly differentiated neurons suggest a possibility that varying amounts of SC1 protein regulate the levels of expression of pro-mitotic genes during cortical development and in this way may indirectly influence the mode of NSC division. We therefore investigated whether we can detect the down-regulation of SC1 and PRMT5 protein levels in the developing cortex at the time when the NSCs switch from proliferative to neuron-generating divisions. We observed that, SC1 protein levels and to a lesser extent PRMT5 protein levels were reduced in the developing cortex at E12 and at E13.5 when symmetric proliferative divisions give way to asymmetric neurogenic divisions (Fig. 7A, B, C and D).

Moreover, we detected a moderate increase in the levels of cycB1 protein in the developing cortex at these developmental stages (Fig. 7E). We conclude that the expression level of SC1 and PRMT5 proteins is down-regulated during the transition from proliferation to neurogenesis of the cortical NSCs concomitant with the elevation of cycB1 protein expression.

## DISCUSSION

In this study we investigated the role of the transcription factor SC1 in neural development. We demonstrated that SC1 expression is dynamically regulated in developing NSCs, being strongly expressed in proliferating NSCs but down-regulated at the onset of neurogenesis. Moreover, experimental knock-down of SC1 in NSCs led to precocious neurogenesis. Notably, we demonstrated that SC1 recruits an epigenetic modifier, the histone arginine methyltransferase PRMT5 and that high levels of SC1/PRMT5 complex are required to maintain the proliferative capacity and “stem-like” cellular state of the NSCs. Furthermore, we showed that SC1 in complex with PRMT5 directs H4R3me2s, a modification which is prevalent in the developing neuroepithelium during the expansion phase of cortical development (35). In addition, we demonstrated that the SC1/PRMT5 complex modulates the levels of expression of pro-mitotic genes that regulate the G2/M transition and mitotic progression.

Our findings suggest that SC1 in complex with the epigenetic modifier, PRMT5, plays an important role in the control of timing of neurogenesis in developing cortical NSCs. Previous work showed that SC1 mRNA is highly expressed in the developing mouse cerebral cortex (34). We found that in E10.5 mouse cortical cell cultures Nestin-positive NSCs expressed variable amounts of SC1 protein. It is not clear whether this reflects heterogeneity within the NSC population, different developmental stages of the NSC lineage(s) or varying levels of SC1 expression during the stages of the cell cycle. Nevertheless, the fact that SC1 expression is low in early-born neurons and that SC1 knock-down in NSCs triggers precocious neuronal differentiation of a subset of the NSCs suggest that NSC differentiation depends on down-regulation of SC1. Consistent with this, we see high levels of

SC1, PRMT5 and H4R3me2s in the early proliferative neuroepithelium at E10.5, while both SC1 and to a lesser extent PRMT5 protein levels are diminished in the neuroepithelium at E13.5 at the onset of neurogenesis. Moreover, we also find that over-expression of SC1 in NSCs isolated from E10.5 mouse cortices leads to a moderate increase in the number of Nestin-expressing NSCs, suggesting that high levels of SC1 prevent differentiation. It is noteworthy in this respect that in the mouse embryonic stem (ES) cells, one of the essential regulators of “stemness”, Nanog, exhibits fluctuating levels of expression and that ES cells which express low levels of Nanog are predisposed to differentiate while those with high Nanog levels retain their pluripotency (43). Perhaps, the low and high SC1-expressing NSCs which we observe within the NSC population reflect a similar heterogeneity of these cells with respect to their predisposition to differentiate.

The dynamic nature of expression of both SC1 and PRMT5 is evident at E15.5 in the developing cortex when both proteins become upregulated in post-mitotic neurons (34) and A.C., unpublished observations. Similarly, another PRDM family member, Blimp1/PRDM1, has been reported to undergo temporally dynamic expression during development of primordial germ cells (PGCs) and various other tissues (44,45) and has also been shown to recruit PRMT5 during PGC development (25). Importantly, various levels of Blimp1 appear to be necessary to direct differentiation of different tissues, reflecting precise dose dependency of different cell types on Blimp1 requirement (45). These observations highlight the general principle of utilising the same transcriptional regulators during development in various tissues in a graded manner to specify different cell fates, possibly through recruitment of different partner proteins and downstream choice of gene targets. The similarities between the utilisation of both Blimp1 and SC1 proteins during development presumably reflect analogous functions of these proteins in different cell lineage precursors during development. For example, the changing cell cycle parameters and kinetics during precursor cell differentiation may be a common mechanism which contributes to the developmental decisions made by these cells (16-19). Precise control of cell cycle progression is one of the critical components of



precursor cell differentiation and changes in the cell cycle parameters are likely to regulate various aspects of the responsiveness of these cells to extracellular signals. Moreover, the observation that both SC1 and PRMT5 are found at high levels in the developing OLP's where PRMT5 has been shown to be necessary for OLP differentiation (this paper and (17)) further underscores the principle of utilizing the same transcription factors in a graded manner throughout development to direct different developmental outcomes.

We observe that SC1 and PRMT5 complex is involved in the down-regulation of *cycB* and *Bub1b* genes. The observation is of interest as previous investigation of the mechanisms responsible for asymmetric partitioning of cell fate determinants during neuroblast divisions in *D. melanogaster* has identified the *cdc2/cycB* complex as instrumental in regulating this process. High levels and activity of these classical regulators of mitosis were found to be necessary for asymmetric division of the neuroblasts during development (19,20). In this respect our finding of diminished expression of both SC1 and to a lesser extent PRMT5 in the developing cortex at E12 and E13.5, when the NSCs switch from symmetric proliferative divisions to asymmetric neuron-generating ones is important. It suggests the possibility that SC1/PRMT5 complex might regulate the timing of neurogenesis in the cortical NSCs by fine-tuning the expression levels of pro-mitotic genes. High levels of SC1/PRMT5 complex found in the pre-neurogenic cortex at E10.5 may keep the expression of pro-mitotic genes *cycB* and *Bub1b* low and might therefore favour symmetric divisions (20), indirectly inhibiting neuronal differentiation – a possibility which should be tested in future investigations. That this might be a more general role of PRDM family members is suggested by the fact that Blimp1, a critical regulator of primordial germ cell (PGC) development, has also been shown to down-regulate pro-mitotic genes (46). Blimp1, like SC1, favours the preservation of the undifferentiated cellular state in PGCs and, also like SC1, exerts its action through recruitment of PRMT5 (25).

PRMT5 is emerging as a critical regulator of cellular “stemness”. Its role in preserving the less differentiated cellular state has been demonstrated in PGCs, erythrocyte progenitors

and ES cells (25,41,46,47). We now provide evidence that PRMT5 is also expressed in developing NSCs during their “stem-like” proliferative stage of development. Together, these observations suggest a fundamental role for PRMT5 and its cognate modifications, H4R3me2s and H2AR3, in maintaining the less differentiated state of stem/progenitor cells in a variety of cell lineages. An intriguing aspect of PRMT5 biology is its dynamic subcellular localization and the recent observation that during ES cell development PRMT5 mediates methylation of R3 on cytoplasmic histone H2A, so preserving ES cell stemness (41). We also detect high levels of both SC1 and PRMT5 in the cytoplasm of neuroepithelial cells in the developing cerebral cortex, suggesting that SC1/PRMT5 might methylate newly synthesized cytosolic histones during S phase, prior to the association of new histones with replicated DNA. Moreover, we find that overexpression of SC1 protein lacking its zinc finger domain, which is exclusively cytosolic and binds PRMT5 very strongly, induces the highest increase in the number of Nestin-expressing NSCs (AC, unpublished observations), further supporting the importance of cytosolic localisation of both proteins in the preservation of cellular “stemness”. A recent report also highlighted the role of PRMT5 in modulating the responsiveness of different cell types to differentiation- or proliferation-inducing growth factors. Intriguingly, high PRMT5 activity was sustained by proliferation-inducing growth factors that favour symmetric divisions, whereas differentiation-inducing growth factors dampened PRMT5 activity leading to cellular differentiation (48).

Regarding the role of histone arginine methylation during cellular development, we recently showed that post-mitotic neurons are marked by a different modification – *asymmetric* dimethylation - of precisely the same arginine residue (H4R3) that is targeted for *symmetric* dimethylation by PRMT5 (35). The asymmetric modification (H4R3me2a) is mediated by PRMT1, a type I arginine methyltransferase (39). While the symmetric dimethylation of arginine by PRMT5 is mainly associated with transcriptional repression, the asymmetric dimethylation mediated by PRMT1 leads to transcriptional activation (39). Since both PRMT5 and SC1 have been found in association with HDAC1 and HDAC2 (33,39,40), it is

possible that SC1 might be a common component of the repressive chromatin remodelling complexes during early neural development, and that the principal role of SC1 in such complexes might be to provide targeting specificity via its sequence-specific DNA binding properties. It is therefore conceivable that down-regulation of SC1 at the onset of neurogenesis has the effect of vacating sites in chromatin that were previously targets of symmetric methylation (repression) by SC1/PRMT5, making them accessible for asymmetric methylation (activation) by PRMT1. This is consistent with our observation that knockdown of SC1 in developing NSCs induces precocious neurogenesis, as it might allow PRMT1-mediated deposition of H4R3me2a modifications leading to the activation of genes necessary for neuronal differentiation. It is also noteworthy in this respect that previous work has identified a protein, Tis21/Btg2 - a known stimulator of PRMT1 activity - as a marker of NSCs that are undergoing their final mitosis on their way to becoming post-mitotic neurons (18). Moreover, it was previously shown that in PC12 cells, which can respond to NGF by differentiating into sympathetic-like neurons, application of NGF increases asymmetric arginine dimethylation of proteins mediated by PRMT1 (49,50). Taken together, these observations suggest that NSC division and neurogenesis is at least partly regulated by the

sequential activation of PRMT5 and PRMT1; high levels of SC1/PRMT5 protein complex during the proliferative stage of cortical development might control the onset of neurogenesis by controlling the cell cycle parameters of the developing NSCs, possibly by maintaining symmetric proliferative divisions of the NSCs during the early phase of cortical development, whereas the progression to asymmetric division and neuronal differentiation depends on PRMT1.

In conclusion, our study identifies SC1 as a modulator of the NSC developmental programme that acts through recruitment of a histone arginine methyltransferase, PRMT5. Given that SC1 is a p75NTR interacting protein (Chittka et al., 2004), it will be important to determine whether Neurotrophins or other signalling molecules can trigger modifications of SC1 that regulate its ability to recruit PRMT5 and thereby transmit extracellular information to the nuclear interior. Perhaps such differentiation-inducing factors as the Neurotrophins regulate which epigenetic modifiers will be recruited by SC1 at different stages of cortical development and regulate the activity of PRMTs involved in the process of neuronal differentiation. Together, our findings uncover a novel role for histone arginine methylation in the control of cortical NSC proliferation and differentiation.

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**ABBREVIATIONS:** BrdU – bromodeoxyuridine, PRMT – protein arginine methyltransferase, SC1 – Schwann cell factor 1, NSC – neural stem cell

## FIGURE LEGENDS

**FIGURE 1.** Dynamic expression of SC1 in developing NSCs. (A-C) NSC cultures immunolabelled with anti-SC1 (left), anti-Nestin, anti-TuJ1 or O4 (centre), and merged with Hoechst DNA stain (right). (A) 3 hours after plating NSCs show different levels of expression of SC1 in Nestin-expressing precursor cells: *arrowheads*, cells with high SC1 expression and *arrows*, cells with low SC1 expression. (B) At 3 DIV differentiating neurons activate TuJ1 expression and down-regulate SC1 (*arrowhead*, high SC1-expressing cell; *arrow*, TuJ1+ neuron with low-level SC1 expression). (C) After 10 DIV high levels of SC1 expression are detected in O4+ oligodendrocyte precursors. (D) Quantification of the percentage of NSCs with high SC1 expression levels. Scale bar, 10  $\mu$ m.

**FIGURE 2.** siRNA knock-down of SC1 leads to precocious differentiation of NSCs into neurons. (A) Western blots of protein samples from rat NSCs transfected with: 1) no siRNA, 2) control (scrambled sequence) siRNA, 3) SC1 siRNA-1, 4) SC1 siRNA-2, probed for expression of endogenous SC1. Bottom panel – same blots probed for actin demonstrate similar protein levels in the designated lanes. (B) NSCs co-transfected with an EGFP expression vector together with either control siRNA (above) or SC1-specific siRNA (below) were immunolabelled with anti-SC1 and anti-EGFP to identify transfected cells. (C) Expression of TuJ1 in NSCs transfected with EGFP and siRNA-1 or EGFP and control siRNA. (Right) Merged images with Hoechst-stained DNA. Two of the neurite-bearing EGFP+ cells from the siRNA-1 treated cultures are magnified to visualise the extensive arborisation. (D) Human or rat SC1 was overexpressed in HEK293T cells with or without rat-specific SC1siRNA-1 or SC1siRNA-2 (sir-1 and sir-2, respectively) and its expression monitored by probing Western blots of transfected cell lysates with anti-SC1. Neither of the applied siRNAs affected the expression of human SC1 (top panel), attesting to the target specificity of the siRNAs. Blots were also probed with anti-tubulin antibodies to control for gel loadings (bottom panel). Co-transfection of ratSC1 cDNA with rat SC1siRNA-2 resulted in the expected reduction in the levels of rat SC1 protein (sir-2/ratSC1 lane). (E) Quantification of TuJ1 expression in NSCs transfected with either control siRNA, siRNA-1 or siRNA-2 or indicated control siRNA [mean  $\pm$  s.d., n=3, *t*-test,  $P < 0.0005$ (\*)]. At least 300 transfected cells were counted per coverslip. (F) Quantification of BrdU incorporation into rat NSCs after transfection with control siRNA, siRNA-2, EGFP alone or nothing (bottom panel). Cells expressing EGFP and indicated siRNA's were immunolabelled to detect BrdU incorporation and EGFP expression (top panel). Scale bars: 100  $\mu$ m (C), 10  $\mu$ m (B and F).

**FIGURE 3.** Immunoprecipitated SC1 complex exhibits an H4 HMTase activity. (A) Myc-tagged SC1 or indicated controls were expressed in HEK293T cells and immunoprecipitated (IPed) using an anti-Myc antibody (below). The same IPs were used for an in vitro HMTase assay with purified calf thymus histone mix; a fluorogram of the in vitro methylation reaction is shown (above, F). (B) Cells were transfected with mycSC1 expression vector or empty Myc vector and cell lysates were immunoprecipitated with anti-Myc antibody. The mycSC1 immunoprecipitate methylates recombinant H4 in an in vitro methylation reaction. F – fluorogram of the methylation reaction products, cbb – coomassie brilliant blue stained membrane showing the histones used for the in vitro methylation reaction. (C) mycSC1 mediated histone methylation increases the levels of H4R3me2s. Same procedure was carried out as in (A) and the blots of methylated histones were probed with antibodies against lysine and arginine modifications. (Panel 1) fluorogram of methylated histones in the indicated IPs, (Panel 2) the same blot probed with anti-H4R3me2s antibody, (Panel 3)

the same blot probed with anti-pan Lysine antibody and (Panel 4) the same membrane stained with Coomassie brilliant blue (cbb).

**FIGURE 4.** SC1 recruits PRMT5 to direct H4 methylation. (A) mycSC1 or empty vector was expressed in HEK293T cells as indicated on the panel. Anti-Myc IPs from transfected cells were analyzed using anti-PRMT5 antibody. Endogenous PRMT5 is found in the complex (*arrow*, top) with IPed mycSC1. Total protein inputs and IPed mycSC1 are shown (two middle and bottom panels, respectively). (B) Endogenous PRMT5 was IPed from HEK293T cells transfected with mycSC1 or empty vector and the IPs were analyzed by Western blot with anti-Myc (*arrow*, MycSC1 protein); two middle and bottom panels show the input mycSC1 and PRMT5 and IPed PRMT5, respectively. (C) Diagram of the deletion constructs of Myc-tagged SC1 used to map the domains of interaction with PRMT5. (D) The NH-terminus and to a lesser extent PR/SET domain of SC1 bind PRMT5. Indicated Myc-tagged SC1 full length or deleted constructs and HA/Flag-tagged PRMT5 were co-expressed in HEK293T cells. Anti-HA IPs (PRMT5) were probed on Western blots with anti-Myc antibody to detect co-IPed myc-tagged SC1 constructs. Inputs were analyzed using anti-Flag antibody for PRMT5 and anti-Myc antibody for SC1. Anti-tubulin antibody was used as a loading control. mycSC1 proteins that co-IP with PRMT5 are highlighted by asterisks on the top panel of the Western blot. (E) mycSC1FL, mycSC1dNH or empty vector were transfected into HEK293T cells. Anti-Myc IPs were used for in vitro HMTase assays. (Above) fluorogram (F) of histones methylated by the indicated IPed complexes, (below) Western blot of IPed proteins used for the in vitro methylation reactions, probed with anti-Myc.

**FIGURE 5.** PRMT5, SC1 and H4R3me2s are expressed in developing NSCs and cortical neuroepithelium and can be co-immunoprecipitated from E10.5 cortex. (A) Expression of PRMT5 in E10.5 Nestin+ NSCs, 3 hours after plating, was detected by immunolabelling with PRMT5-specific antibodies. Right panels, merged images with Hoechst DNA stain. (B) H4R3me2s modification is detected in Nestin+ NSCs 3 hours after plating. (C) Both SC1 and PRMT5 are expressed in the developing mouse cortex at E10.5 and high levels of H4R3me2s modifications are detected in the cortical neuroepithelium at this stage. Expression of the relevant proteins or modification in panels (A, B and C) was detected by immunolabelling with anti-SC1, anti-PRMT5 or anti-H4R3me2s. (D) Endogenous PRMT5 was IPed from E10.5 cortices and the presence of endogenous SC1 in the co-IPed complex was analyzed by using anti-SC1 antibodies. Non-specific IgG was used to control for the specificity of IP reactions. Input and IPed PRMT5 is shown on the middle panel and anti-actin antibody was used as a loading control. Scale bars: 10  $\mu$ m (A, B), 50  $\mu$ m (C). V, ventricular zone; P, pial surface.

**FIGURE 6.** SC1 and PRMT5 complex increases the number of Nestin-expressing neural precursors and regulates expression of pro-mitotic genes. (A) Over-expressed mycSC1 increases the number of Nestin+ NSCs. mycSC1FL and mycSC1dNH proteins were detected by immunolabelling with anti-myc antibodies and NSCs by the presence of Nestin immunoreactivity. Quantification of Nestin+/mycSC1 expressing NSCs is shown in the graph on the right. At least 300 cells were counted per transfection and data are shown as mean  $\pm$  s.d [n=3, p<0.05(\*)]. (B) Semi-quantitative RT-PCR was used to estimate the relative levels of *cycB* and *Bub1b* mRNA in P19 cells transfected with mycSC1FL, mycSC1dNH or empty vector. (C) Semi-quantitative RT-PCR was used to estimate the relative levels of *SC1*, *cycB* and *Bub1b* mRNA in PC12 cells transfected with SC1 siRNA, control siRNA or no siRNA. Levels of mRNA were normalized to *GAPDH* mRNA. Scale bar: 5  $\mu$ m (A).

**FIGURE 7.** SC1 and PRMT5 protein levels are reduced in the developing cortex at E12 and E13.5. Mouse cortices from E12 (A) and E13.5 (B) embryos were immunolabelled for TuJ1 and SC1. SC1 protein levels are reduced compared to those detected at E10.5 prior to the onset of neurogenesis (see Fig. 5). Bright red signal in the tissue represents non-specific labelling of blood vessels after antigen retrieval by heating with citrate buffer. (C) Western blot analysis of SC1 protein expression in the developing cortex. Protein homogenates from embryonic cortices of indicated ages were analysed by probing with anti-SC1 antibodies (top panel) and anti-actin (bottom panel) antibodies to control for

protein loading. Normalised protein levels of SC1 are shown in the graph. (D) Mouse cortex from E13.5 embryos was immunolabelled for TuJ1 and PRMT5. Moderate levels of PRMT5 protein were detected in the cortex at E13.5. TuJ1 staining is towards the pial surface in all panels. (E) Western blot analysis of cycB1 protein expression in the developing cortex. Protein homogenates from embryonic cortices of indicated ages were analysed by probing with anti-cycB1 antibodies (top panel) and anti-actin (bottom panel) antibodies to control for protein loading. Data (in C) are shown as mean  $\pm$  s.d from three independent western blot quantifications [n=3, p<0.05(\*)]. Scale bar, 75  $\mu$ m (top panels), 25  $\mu$ m (bottom panels).















