The RNA-binding protein Musashi-1 is produced in the developing and adult mouse eye


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Purpose: Musashi-1 (Msi1) is an RNA-binding protein produced in various types of stem cells including neural stem/progenitor cells and astroglial progenitor cells in the vertebrate central nervous system. Other RNA-binding proteins such as Pumilio-1, Pumilio-2, Staufen-1, and Staufen-2 have been characterized as potential markers of several types of stem or progenitor cells. We investigated the involvement of Msi1 in mouse eye development and adult mouse eye functions by analyzing the profile of Msi1 production in all ocular structures during development and adulthood.

Methods: We studied Msi1 production by in situ hybridization and immunohistochemistry of ocular tissue sections and by semi-quantitative RT-PCR and western blot analysis from the embryonic stage of 12.5 days post coitum (E12.5 dpc) when the first retinal ganglion cells (RGCs) begin to appear to the adult stage when all retinal cell types are present.

Results: Msi1 mRNA was present at all studied stages of eye development. Msi1 protein was detected in the primitive neuroblastic layer (NbL), the ganglion cell layer (GCL), and in all major differentiated neurons of postnatal developing and adult retinae. During postnatal developing stages, faint diffuse Msi1 protein staining is converted to a more specific distribution once mouse retina is fully differentiated. The most striking result of our study concerns the large amounts of Msi1 protein and mRNA in several unexpected sites of adult mouse eyes including the corneal epithelium and endothelium, stromal keratocytes, progenitor cells of the limbus, equatorial lens stem cells, differentiating lens epithelial cells, and differentiating lens fibers. Msi1 was also found in the pigmented and nonpigmented cells of the ciliary processes, the melanocytes of the ciliary body, the retinal pigment epithelium, differentiated retinal neurons, and most probably in the retinal glial cells such as Müller glial cells, astrocytes, and the oligodendrocytes surrounding the axons of the optic nerve. Msi1 expression was detected in the outer plexiform layer, the inner plexiform layer, and the nerve fiber layer of fully differentiated adult retina.

Conclusions: We provide here the first demonstration that the RNA-binding protein, Msi1, is produced in mouse eyes from embryonic stages until adulthood. The relationship between the presence of Msi1 in developing ocular compartments and the possible stem/progenitor cell characteristics of these compartments remains unclear. Finally, the expression of Msi1 in several different cell types in the adult eye is extremely intriguing and should lead to further attempts to unravel the role of Msi1 in cellular and subcellular RNA metabolism and in the control of translational processes in adult eye cells particularly in adult neuronal dendrites, axons, and synapses.

RNA-binding proteins (RBPs) have been shown to play a major role in the regulation of mRNA translation and localization. The roles of RBPs have been and are still extensively studied in early development. Recent studies from several groups have demonstrated that RBPs, which associate with active polysomes, play a crucial role in the translational regulation of neuronal mRNAs, determining neuronal polarity [1,2]. Electron microscopy studies have demonstrated that polysomes are present in the dendrites, axons, and growth cones of developing neurons [3-5]. These studies have shown that the mRNA molecules are clustered into polysome-containing RNA protein (RNP) granules with RBPs such as Fragile X Mental Retardation Protein (FMRP), Zipcode Binding Protein 1 (ZBP1), and Staufen [6,7]. Following transcription, specific regulatory proteins attach to the mRNA molecule and regulate its exit from the nucleus, cytoplasmic transport, distribution, and translation. The regulation of mRNA is particularly crucial during neuronal cell differentiation when pluripotent precursor cells differentiate into neurons. Differentiation increases the transcription of a multitude of genes as recently reported for the P19 embryonic carcinoma cell line [8]. Several studies have addressed the role of RBPs in the regulation of mRNA transport, anchoring, and translation in neuronal cells [9]. However, only a few mammalian RBPs have been studied extensively in the context of ocular development, in the adult eye and during the adult retinal functioning.

Musashi protein (Msi) was initially isolated in Drosophila as a molecule required for the asymmetric division of sensory organ precursor cells [10,11]. Vertebrates have two members of the Musashi family, Msi1 and Msi2, which are highly conserved across species [10,12-15]. These proteins contain two RNA recognition motifs (RRMs). The identity and role of the RNA targets of Msi1 and Msi2 in vivo remain largely unknown. However, it has been demonstrated in mammals that Numb, a
key regulator of neural proliferation and differentiation that acts by repressing Notch, is regulated by Msi1 at the translational level [16]. Another potential target of Msi1 has been identified as a cyclin-dependent kinase inhibitor, p21WAF1 [17], which is consistent with a role for Msi1 in cell cycle regulation and differentiation.

Msi1 and Msi2 are produced predominantly in proliferating embryonic neural precursors and neural stem cells [11,18-20]. Msi1 is highly abundant in neural stem/progenitor cells, astroglial progenitor cells, and astrocytes in the developing and adult central nervous system (CNS) [18,21,22]. This protein plays an important role in regulating the differentiation of precursor cells and seems to be involved in the self-renewal and maintenance of CNS stem cell populations [23]. Msi1 is not restricted to neuronal stem cells, it is also found in other types of stem/progenitor cells and in different species, suggesting that this protein may be a general stem cell marker. For instance, Msi1 has been recognized as a distinctive marker of epithelial stem cells in the crypt of the mouse small intestines and the human colon [24,25]. Msi1 has also been found in mouse stem cells in the bulge region of the hair follicle [26]. The diversity of stem cell populations containing Msi1 protein also suggests that Msi is involved in a general mechanism for regulating stem cell maintenance and differentiation.

Asymmetric cell division (ACD) is a fundamental mechanism for generating cellular diversity in invertebrates and vertebrates. During mammalian retinal development, neurons and glial cells are generated from multipotent retinal progenitor cells [27]. Cell divisions giving rise to differentiating cells initially occur only in the central retina [28,29]. Concomitant cell divisions in the peripheral retina are exclusively symmetric and essential for increasing the pool of progenitor cells [30]. Retinal cell types are generated in a chronological sequence conserved throughout evolution. Most of the retinal ganglion cells (RGCs), horizontal cells, amacrine cells, and cone photoreceptors are generated during early histogenesis whereas most rods, bipolar cells, and the Müller glia are generated during late histogenesis [31].

The amphibian retina continues to grow and new cells are continually added in the stem cell-containing zone, a region known as the ciliary marginal zone (CMZ) [32,33]. The presence of potential retinal stem cell population within the ciliary epithelium of birds has been reported [34,35]. In mammals, however, retinal neurogenesis is completed shortly after birth and there is no evidence for a peripheral retinal growth zone (for review, [36]). Nevertheless, even in adult mice, the ciliary body contains stem cells capable of generating retinal neurons including photoreceptor cells in vitro [37,38]. Moreover, other studies have identified the presence of a quiescent mitotic population of cells in the peripheral margin of the postnatal mammalian retina [39] and ciliary epithelium [40].

The stem cells in the mammalian retina have not been well-defined due partly to the lack of very specific molecular markers and partly due to the very small numbers of these cells. To date, Msi1 homologs have been found only in retinal stem cells, mitotically active neural precursors, postmitotic photoreceptors, and retinal pigment epithelium (RPE) cells during retinal development in *Xenopus* [41] and in the photoreceptor cell nuclei in *Drosophila* [13].

As part of a continuing investigation of the role of RBPs in eye development, we examined the expression of Msi1 and other RBPs (Musashi 2 [Msi2], Pumilio 1 [Pum1], Pumilio 2 [Pum2], Staufen 1 [Stau1], and Staufen 2 [Stau2]) in rodent eyes. We found that only Msi1 was produced in significant quantities in the eye from embryonic stages until adulthood. Msi1 was produced in various ocular cell types in both proliferating and differentiated cells. This pattern is largely maintained during adulthood. These results suggest that Msi1 is involved in eye development and may have multiple molecular targets involved in the biological functions associated with cell proliferation and differentiation at various stages of development. They also indicate that Msi1 probably plays multiple roles in maintaining the differentiation state and in the functions of several ocular cell types.

**METHODS**

**Tissue preparation:** C57Bl6/J mice, used for the preparation of tissue RNA extracts or tissue sections, were obtained from Charles River (L’Arbresle, France). The date of conception was established by the presence of a vaginal plug and recorded as E0.5 and the day of birth was designated as postnatal day 0 (P0).

The embryos were microdissected from the whole tropheoblast and placed on the surface of hard plastic cups filled with an optimal cutting temperature (OCT) medium (Tissue Tek; Bayer Diagnostic, Puteaux, France). The lower surface of the cups was then carefully placed in contact with the surface of a progressively refrigerating isopentane solution. The cups were maintained at the surface of the refrigerating isopentane solution until a temperature of -30 °C was reached. The specimens were subsequently frozen in powdered dry ice for 15 min and then stored at -80 °C until use. Eyes were obtained from eight-week-old mice and treated according to the same procedure. Cryostat sections (14 μm thick) were mounted on slides coated with 2% 3-aminopropyl-triethoxylane in acetone. Sections were fixed by incubation for 30 min in 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), rinsed once in phosphate-buffered saline then briefly in water, and dehydrated by incubation in a series of graded alcohol solutions. Sections were then air dried and stored at -80 °C. This procedure was used to preserve mRNA in embryonic and fetal tissues.

Immature animals (P1 to P12) and adult mice (P30 and P60) were killed by CO2 asphyxiation. Eyes were rapidly enucleated and fixed by incubation for at least 36 h in 4% paraformaldehyde (PFA) at 4 °C. They were embedded in paraffin and cut with a microtome (HM355; Microm, Les Ulis, France) into 5 μm sections, which were mounted on glass slides (Superfrostplus; Fisher Scientific, Illkirch, France), dried overnight at 37 °C, and stored at room temperature until use.

**DNA probes for in situ hybridization:** The Msi1 probes were based on the mouse Msi1 cDNA sequence (GenBank: NM_008629). The sequences of the Msi1 probes were: Antisense Msi1: 5'-CTT AGG CTG TGC TCT TCG AGG...

AAA GGC CAC CTT GGG GTC AAT TGT TTT GGA GTC GAG CTC -3' (position 372-313) and Sense Msi1: 5'- GAG CTC GAC TCC AAA ACA ATT GAC CCC AAG GTG GCC TTT CCT CGA AGA GCA GAC CAG CCT AAG -3' (position 313-372). The Msi2 probes were based on the mouse Msi2 cDNA sequence (GenBank NM_050403). The sequences of the Msi2 probes were: Antisense Msi2: 5'- ATT TAT CTG GTG AGG TCT GCC AGC TCA TCA GAT ACC ATT AGG GGC ACC ACA AAT -3' (position 3690-3631) and Sense Msi2: 5'- CCC AGC ACG ACC CGG GTA AAA TGT TTA TCG GTG GAC TGA GCT GGC AGA CCT CAG CAC ATA -3' (position 186-245).

The Pum1 probes were based on the mouse Pum1 cDNA sequence (GenBank NM_030722). The sequences of the Pum1 probes were: Antisense Pum1: 5'- GTG AGC GGG ACA GGG AGC TGG GTG CAC TGT CTA GAT ACC ATT AGG GGC ACC ACA AAT -3' (position 3690-3631) and Sense Pum1: 5'- ATT TAT CTG GTG CCC CCT AAT GAT ATC ATC TGA ACA GTG CCC CCA CCT CCC TGG CCT GGC GCT GAC -3' (position 3631-3690). The Pum2 probes were based on the mouse Pum2 cDNA sequence (GenBank NM_030723). The sequences of the Pum2 probes were: Antisense Pum2: 5'- GGG GGC GGG AGG GGA GGA CAC AGT TAC ATC ATA TAC AGG CAT TCT GTG CTT CAT CAG CAA AAA -3' (position 3664-3605) and Sense Pum2: 5'- TTT CTG GTG AAG CAC AGA ATG CCT GTA TAT GAT GTA ACT GTG TCC CCC CCT CCC GGC CCC -3' (position 3605-3664). The sense probes were used as a negative control.

The 60-mer oligonucleotide probes were synthesized and purified by Eurogentec (Angers, France). The oligonucleotides were 3'-end labeled with 35 S dATP (PerkinElmer, Courtabeuf, France), using terminal deoxynucleotidyl transferase (15 U/ml; Invitrogen, Cergy-Pontoise, France) at the highest possible magnification. The tissues were frozen in liquid nitrogen. Total RNA (1 µg) was reverse-transcribed, using an oligoT primer with SuperScript II RNase H reverse transcriptase (Invitrogen) in a total reaction volume of 20 µl.

The products of reverse transcription (1 µl) were amplified by PCR in a reaction volume of 10 µl containing 0.25 µM of each primer, 0.5 U Taq DNA polymerase (Invitrogen), 10X PCR buffer, 2 mM MgCl₂, and 0.2 mM dNTP (Promega, Charbonnières-les-Bains, France).

Msi1 primers (forward 5'-GTT CAT CGG AGG ACT CAG-3' and reverse 5'-GCT CTC AAA CTC AAC CGG GTG AAG AA-3') were designed so as to amplify a 411 bp fragment. Msi2 primers (forward 5'-TGC TGC GAA CAG AGT ATG AGT GAA A-3' and reverse 5'-GTA GCC TCT GCC CCA ATA GTG GTC A-3') were designed so as to amplify a 339 bp fragment. Pum1 primers (forward 5'-TGT ACT TTC CCC ACG GTC GG-3' and reverse 5'-CGG GAG CTA AAC CTG CGA TG-3') were designed so as to amplify a 649 bp fragment. Pum2 primers (forward 5'-TTC CAC AGC CAA GAG ACG CA-3' and reverse 5'-GCA TTC CTC AGG CAC CAC AGC AG-3') were designed so as to amplify a 569 bp fragment.

The cyclophilin primers were based on the mouse cyclophilin cDNA sequence (GenBank NM_008907). Cyclophilin primers (forward 5'-TGG TAC CCA CCG TGT TCT TC-3' and reverse 5'-TCC AGC ATG GAC CTT GCT A-3') were designed so as to amplify a 311 bp fragment (Invitrogen). Cyclophilin was coamplified with the target gene as an internal control for comparative purposes.

PCR amplification was performed as follows for Msi1: 95 °C for 10 min, 40 cycles of 94 °C for 30 s, 55 °C for one min, and 72 °C for one min with a final extension step at 72 °C for seven min; for Msi2: 94 °C for three min, 30 cycles of 94 °C for one min, 61 °C for one min, and 72 °C for one min

**In situ hybridization procedure:** The hybridization cocktail contained 50% formamide, 4X SSC (standard saline citrate), 1X Denhardt’s solution, 0.25 mg/ml yeast RNA, 0.25 mg/ml sheared herring sperm DNA, 0.25 mg/ml poly(A)+, 10% dextran sulfate (Sigma-Aldrich, Saint-Quentin Fallavier, France), 100 mmol DTT (dithiothreitol) and 35 S dATP-labeled probes (6x10⁶ cpm/100 µl, final concentration). We applied a coverslip, and examined under bright- or dark-field illumination with a DNRB2 light microscope (Leica, Rueil-Malmaison, France). Both bright and dark-field images were collected by a charge-coupled device (CCD) camera (Nikon, Tokyo, Japan) connected to a computer.

**RNA extraction and reverse transcription-polymerase chain reaction:** Total RNA was extracted from mouse eyes at different postnatal ages (P0, P8, P15, P21, P28, and P60, n=3) using the TRIzol® reagent (Invitrogen, Cergy-Pontoise, France) according to the manufacturer’s recommendations. RPE cells were taken out according to the following procedure of the swift death of the mice used for microdissection then a fast enucleation. The cornea, iris, and lens were quickly removed with scissors and forceps, and the neural retina was taken out. The RPE monolayer was swiftly isolated by scraping with a small spatula. The whole microdissection procedure was performed under biomicroscope (ZEISS, Le Pecq, France) at the highest possible magnification. The tissues were frozen in liquid nitrogen. Total RNA (1 µg) was reverse-transcribed, using an oligoT primer with SuperScript II RNase H reverse transcriptase (Invitrogen) in a total reaction volume of 20 µl.
with a final extension step at 72 °C for seven min; for Pum1: 94 °C for three min, 30 cycles of 94 °C for one min, 64 °C for one min, and 72 °C for one min with a final extension step at 72 °C for seven min; for Pum2: 94 °C for three min, 30 cycles of 94 °C for one min, 62 °C for one min, and 72 °C for one min with a final extension step at 72 °C for seven min. Stau1: 94 °C for 3 min, 40 cycles of 94 °C for one min, 64 °C for one min, and 72 °C for 1 min with a final extension step at 72 °C for seven min; for Stau2: 94 °C for three min, 30 cycles of 94 °C for one min, 59 °C for one min, and 72 °C for one min with a final extension step at 72 °C for seven min; and for cyclophilin: 94 °C for one min, 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for one min with a final extension step at 72 °C for seven min.

PCR amplification products were analyzed by electrophoresis in 1% agarose gels and visualized by ethidium bromide staining under UV light. The intensity of bands was quantified with Image J software.

**Western blot analysis:** Western blot analysis was performed to determine the specificity of the Msi1 antibody. Total proteins were extracted separately from total eyes and from neuroretinas of adult C57BL/6J mice, using extraction reagent (TRizol; Invitrogen-Gibco, Cergy-Pontoise, France) according to the manufacturer’s instructions. Proteins (100 μg; concentrations determined with the Bradford protein assay) were separated by electrophoresis (in a 10% polyacrylamide gel containing SDS). Proteins were transferred onto a nitrocellulose membrane, which was blocked by incubation with 5% skim milk for 1 h. Membranes were then incubated overnight with the same rabbit anti-Msi1 antibody used for immunohistochemistry experiments (1:200; Chemicon, Paris, France) or a goat anti-β-actin antibody (1:1000; Santa Cruz, Santa Cruz, CA). Membranes were washed and incubated for 2 h with horseradish peroxidase-linked anti-rabbit secondary antibody (Santa Cruz). Proteins were then detected by chemiluminescence (ECL; PerkinElmer Life and Analytical Sciences, Inc., Courtabeuf, France).

**Immunohistochemistry:** Paraffin-embedded sections were incubated in xylene to remove the paraffin and rehydrated by incubation in a graded series of alcohol solutions. The sections were labeled using the detection kit (ChemMate; Dako, Trappes, France) according to the manufacturer’s instructions. A rabbit anti-mouse Msi1 polyclonal antibody was used as the primary antibody (diluted 1:200; Chemicon). The secondary antibody was a biotinylated antibody (ChemMate detection kit; Dako, TRAPPES, France) with diaminobenzidine (DAB) as its substrate. After DAB staining, tissue sections were counterstained with 3% Methyl Green solution (Sigma-Aldrich, Saint-Quentin Fallavier, France).

**RESULTS**

Msi1 mRNA production in embryonic and postnatal mouse eye: Msi1 gene expression in mouse ocular tissues was studied by RT-PCR and radioactive in situ hybridization at several stages of mouse eye development. From E12.5 when the first RGCs are established to E18.5, Msi1 mRNA was detected by radioactive in situ hybridization throughout both the neuro-
week (around P11) [43,44]. We carried out immunohistochemical investigations of the cellular distribution of Msi1 protein in the adult retina and in the developing retina from P1 to P12. Msi1 protein was detected primarily in the GCL and in the outer region of the NbL of the developing retina (Figure 7B,C). Significant immunostaining of the RPE cells was detected during postnatal stages of development (Figure 7B-F). From P8 to P12, Msi1 protein was detected throughout the INL and the ONL with persistent very strong staining for Msi1 protein in the GCL (Figure 7D-F). The intensity of Msi1 immunolabeling in the INL increased over time as the neurorsetina completed its development (Figure 8B-D). All the cell bodies of the ONL, the INL, and the GCL were unambiguously immunoreactive for Msi1 during adulthood (Figure 8B-D). As all the cell bodies of the ONL were immunolabeled for Msi1, it seems highly likely that the soma of rods and cones are both positively immunolabeled for Msi1.

The horizontal cells, which are located very close to the photoreceptors and can be distinguished based on their flattened morphology in the outer plexiform layer (OPL), were also unambiguously immunolabeled for Msi1. Msi1 protein is almost certainly present in the cell bodies of the neuronal bipolar cells, amacrine cells, and Müller glial cells present in the INL. The cell bodies and fibers of neurons located in the inner plexiform layer (IPL) were also unambiguously labeled. Further, double labeling experiments are required to determine whether these neurons are displaced amacrine cells or special classes of displaced ganglion cells. The GCL appeared to display stronger Msi1 immunoreactivity than the cell bodies of the INL and the ONL. The OPL, the IPL, and the nerve fiber layer (NFL) were also positively immunolabeled (Figure 8B-D), suggesting that Msi1 protein may be present in both dendrites and axons. Msi1 protein appears to be abundant not only in all RGC bodies but also in ganglion cell fibers (Figure 8).

Figure 1. Msi1 mRNA production in embryonic mouse eye. A-D: In the retinal labeling observed in a dark field, Msi1 transcripts were detected at high levels in the neuroblastic layers of the retina from E12.5 to E18.5. E-F: A high magnification confirms the presence of Msi1 labeling in the retina in (E) the dark-field image (white grains) and in (F) the bright-field image (black grains). NbL, neuroblastic layer; GCL, ganglion cell layer; RPE, retinal pigment epithelium; Le, lens epithelium cells; Lf, lens fiber, Tz, transitional zone.
corresponding to the axons of the retinal ganglion cells, a major component of the optic nerve. Msi1 proteins are also probably present in retinal astrocytes, which are abundant in the GCL, around the endothelial cells of the outer blood-eye barrier, and in the oligodendrocytes surrounding the RGC axons as columns of aligned Msi1 immunolabeled cell bodies were observed in sections of the optic nerve (data not shown).

In summary, the Msi1 protein is present in early postnatal differentiated retinal cells, predominantly ganglion cells. It is later produced in all adult neuronal retinal cells, in the OPL, in the IPL, and in the ganglion nerve fiber cells when postnatal retinal development approaches adult stages. Msi1 protein is present in all adult retinal neuronal cells and may be present in retinal glial cells.

At high magnifications, in tissue sections of non retinal structures, strong Msi1 immunolabeling was observed throughout the multilayered corneal epithelium. Msi1 immunoreactivity was stronger in the basal mitotic progenitor corneal epithelial cells than in the superficial cells located above them (CEp), the stromal keratocytes, and the corneal endothelium (CEn; Figure 9A-C). The limbal cells including the stem and progenitor cells of the limbus were unambiguously and strongly stained for Msi1 as observed in the transitional zone of the limbus. In this zone, the nonkeratinized multilayered corneal epithelium is reduced to fewer cell layers and the well-structured distribution of stromal collagenic plaques and flattened keratocytes tends to disappear. It is replaced by a dense region which becomes the sclera, the main features of which are rich vascularization, a lack of collagen organization, opacity, and highly protective mechanical properties (Figure 9D,E). Msi1 immunoreactivity was also detected in the two pigmented layers of the iris, the iris stromal melanocytes, and iris mesenchymal cells derived from the neural crest (Figure 9F). It was also observed in the pigmented ciliary epithelial cells (PCE) and nonpigmented ciliary epithelial cells (NPCE) of the ciliary body (Figure 9G). Msi1 protein was produced during development in the lens equatorial epithelial cells of the germinative zone, in the lens epithelium, and in the early differentiating lens fiber cells (Figure 9H). This pattern of expression in these cells was maintained throughout adulthood. Moreover, strong Msi1 immunoreactivity was observed in the adult RPE and in the choroidal melanocytes but not in the choroidal vascular endothelial cells or vascular uveal endothelial cells (Figure 9I).

The presence of Msi1 in all ocular compartments including all the potential regions of ocular stem cells in developing and adult mouse eyes suggests that this protein is involved in several functions during eye development and functioning.

**DISCUSSION**

We used RT-PCR, radioactive in situ hybridization, western blotting, and immunohistochemistry to analyze Msi1 mRNA and protein production in the ocular tissues of developing and adult mice.

**Msi1 production in the embryonic and postnatal developing retina:** The regulation of mRNA translation by RBPs is a key mechanism for controlling temporal and spatial gene expression during many cellular and developmental processes including neural stem cell self-renewal [45]. A recent study in *Drosophila* demonstrated an intrinsic requirement for Musashi to maintain stem cell identity [46]. The balance between stem cell maintenance and differentiation must be tightly regulated, but plasticity is required to maintain tissue homeostasis under fluctuating environmental conditions. We showed that *Msi1* mRNA was present in the neuroblastic layer, which contains early progenitor cells and cells in different stages of proliferation and differentiation. Msi1 was also found in newly differentiated cells of the ganglion cell layers. Our results are consistent with those of a previous study showing that Msi1 is present in neurospheres generated from developing retinal cells [47]. Indeed, Nrp-1, a homolog of mouse Msi1, is produced in both stem cells and mitotic precursors in developing *Xeno-
Msi1 has been shown to function in neural stem cell self-renewal as a repressor of the translation of Numb mRNA [11,48]. The various rodent isoforms of Numb seem to have two different functions in the neuronal lineage, particularly during retinal development [49]. The molecular mechanisms underlying the repression of m-Numb mRNA translation by Msi1 remain to be elucidated. Indeed, Msi1 overproduction activates Notch1 signaling, which regulates cell fate decisions during development [16]. Notch signaling is known to induce the self-renewal of mammalian neural stem cells [50,51]. Moreover, studies in both vertebrate and invertebrate nervous systems have established a critical role for Notch signaling in preserving a pool of undifferentiated progenitor cells (for review, [52]). In the developing retina, Notch signaling seems to be involved in maintaining retinal progenitor cells (RPCs) in an undifferentiated state [53,54].

Figure 3. Expression of Msi1 mRNA in mouse eye during development and at adulthood. Semiquantitative RT-PCR was used to determine the relative amounts of Msi1 mRNA (A) in whole mouse eye at P0, P8, P15, P21, P28, and P60A and (C) in the neuroretina (Re), retinal pigmented epithelium (RPE), and ciliary body (CB) of adult mice. Cyclophilin A was used as an internal control. The 411 and 311 bp bands correspond to Msi1 and cyclophilin PCR products, respectively. B, D: Densitometric analysis of intensities of PCR bands correspond to A and C, respectively. The relative levels were calculated as the ratio of intensities of the Msi1 band to the cyclophilin band. B: The intensity of the Msi1 band increased from P8 to P15 and seemed to be stable from P15 until adulthood. D: Msi1 mRNA levels were higher in the retina than in the RPE and CB (n = 3). Error bars indicate SEM.

Figure 4. Expression of Msi2, Pum2, and Stau2 mRNAs in different compartments of adult mouse eye. RT-PCR was used to determine Msi2, Pum2, and Stau2 mRNA levels in the whole eye (We), neuroretina (Re), retinal pigmented epithelium (RPE), and the ciliary body (CB) of adult mice. The 339, 161, 569, and 311 bp bands correspond to Msi2, Pum2, Stau2, and cyclophilin PCR products, respectively.

pus retina [41]. Nrp-1 is also present in postmitotic photoreceptors and the RPE. These observations suggest that the pattern of Msi1 production in the developing retina has been conserved during evolution.
Based on these data, we hypothesized that Msi1 might be involved in maintaining retinal stem cells and progenitor cells from the earliest stages of retinal development as in other regions of the CNS. The role of Msi1 might not be limited to the repression of mRNA translation during development. Msi1 may also act as a multifunctional regulator, controlling its target genes at several different steps of posttranscriptional regulation including splicing, translation, stability control, and determination of the distribution of mRNAs. However, it remains unclear why and how a single RBP might play a multifunctional role in posttranscriptional gene regulation. The intracellular distribution of Msi1 protein varies (cytoplasmic and/or nuclear) depending on cell-type and/or developmental stage. This suggests that Msi1 may be involved in steps other than controlling RNA translation.

Msi1 production in the adult retina: All retinal layers, ONL, INL, GCL, OPL, and IPL as well as the photoreceptor inner segments (PIS) show strong and specific Msi1 immunoreactivity throughout the adult mouse retina. These results

Figure 5. Msi1 mRNA production in the adult eye. A: In adults, Msi1 mRNA was still detected in the neuroretina with a strong signal observed in the ganglion cell layer, inner nuclear layer, and outer nuclear layer. B: Histological appearance of the same retina observed in bright field at high magnification, making it possible to distinguish the black grains corresponding to labeling. RPE, retinal pigmented epithelium; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; C-D: Msi1 mRNA was also detected in the ciliary body (CB) at the adult stage. The image in D corresponds to that in C in bright field at high magnification, making it possible to distinguish the black grains corresponding to labeling.

Figure 6. Western blot for Msi1 antibody specificity. The whole eye (We) and neuroretina (Re) from adult mice were harvested and lysed then protein was extracted. The western blot was probed with polyclonal antibodies directed against Msi1 and β-actin (internal control). Specific bands for Msi1 (39.1 kDa) and β-actin (35 kDa) were detected in whole eye and neural retina extracts.
Figure 7. Msi1 protein production in the developing mouse retina. Protein was detected by immunochemistry, using a rabbit anti-mouse Msi1 antibody with 3% methyl green counterstaining. Msi1 is present principally in the ganglion cell layer (GCL) and in the outer region of the neuroblastic layer (NbL) of the developing retina (B and C). This pattern persists throughout postnatal stages of development with the intensity of Msi1 immunolabeling in the GCL increasing in later stages of development (D-F). Msi1 is dispersed in the INL and ONL late in retinal development (D-F). A: Immunonegative control. NbL, neuroblastic layer; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.
suggest that all retinal neurons and glial cells display positive immunolabeling for Msi1. Moreover, the positive Msi1 intra-cellular immunoreactivity of neurons is consistent with Msi1 being transported from the retinal nuclei to different subcellular sites where it carries out different functions. Until this study, Msi1 had never been reported to be present in adult retinal neurons. However, Msi1 protein has previously been detected in the spiral ganglion neurons of young adult mice, which play a major role in the physiology of the auditory system [55]. So, what role might Msi1 play in adult retinal neurons?

In this study, we observed that Msi1 immunoreactivity was strongest in the RGC cell bodies. Immunohistochemical staining cannot be considered truly quantitative, but this stronger immunoreactivity in RGC cell bodies probably corresponds to larger amounts of Msi1 protein. These large retinal neurons display intense metabolic activity, correlated with the very high oxygen consumption of the inner retina documented in several studies [56,57]. The probable high concentration of Msi1 in RGCs may be related to one of the main features of these retinal neurons - the integration of all the electrical signals reaching these cells and originating from all the retinal neurons located downstream from them - and to the function of RGCs in transmitting action potentials to the upstream neurons located in the brain. Two other specific RRM RNA-binding proteins, Pum2 and Stau2, have been detected by RT-PCR analysis in the whole eye and retina of adult mouse. Pum2 and Stau2 are required for different functions in various neuronal components. Pum2 is involved in neuronal dendrite morphogenesis via translational control mechanisms [58] and in the dendrite and/or neurite-specific translation of various mRNAs [59,60]. Pum2 has also been shown to regulate neuronal excitability by modulating the expression of a voltage-gated sodium channel [59,61]. Together with Stau2, it is required for long-term memory [62]. Pum2 also influences syn-

Figure 8. Msi1 protein production in adult mouse retina. The intensity of immunolabeling for Msi1 in the INL increased as the neuroretina approached maturity (B-D). The outer plexiform layer (OPL) and the inner plexiform layer (IPL) were both positively immunolabeled for Msi1 (B, D). Msi1 protein was also observed in the photoreceptor inner segment (IS; B, C). Msi1 protein remained abundant in all RGC bodies and in the nerve fiber layer (NFL; D) A: Immunonegative control. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS, inner segment; OS, outer segment; RPE, retinal pigment epithelium.
aptic growth and function by regulating eukaryotic initiation factor 4E (eIF4E) mRNA production at the Drosophila neuromuscular junction [63]. Stau2 plays a crucial role in mRNA trafficking in dendrites [64-68]. The role of neuronal RBPs is not confined to controlling the distribution and translation of various mRNA subsets in specific dendrites. Msi1 may therefore not be limited to the targeting of some mRNAs to specific dendrites by means of the different actions of isolated or combined RBPs but may instead also affect axons or even synapses. This compartmentalization may be a crucial element of the molecular basis underlying complex neural functions. Indeed, the targeting of mRNAs to different functional neuronal domains is essential for memory storage [62]. Therefore, the cooperative actions of the two mRNA-binding proteins direct the distribution of an mRNA encoding a key synaptic protein as described, for example, in the sensory neurons of Aplysia [69]. We have demonstrated that Msi1 protein is present in various compartments of adult photoreceptors: the cell bodies located in the ONL, the PIS, and the OPL, which contains photoreceptor axons synapsing with dendrites of neuronal bipolar cells. Msi1 transport from the photoreceptor cell bodies to the PIS may be governed by specific sets of RBPs including Stau2 and Pum2. Msi1 is highly abundant in all the retinal ganglion cell bodies and ganglion cell fibers thus, in the axons of the RGCs. An important hypothesis arises from these results and deserves to be tested experimentally: Msi1 mRNAs and proteins might be transported from RGC cell bodies to their axons, neurites, and presynaptic extremities. Other RBPs might cooperate with Msi1 in the targeting of mRNA subsets to specific neuronal compartments, dendrites, axon hillocks, neurites, axonal branches, axon terminals, or even synapses. The presence of Msi1 in adult retinal neurons suggests that this RBP may play an important role in the functioning of these neurons. This hypothesis is supported by the targeting of m-Numb by Msi1 as this molecule has been shown to play an important role in neural cell fate determination and differentiation during development [70,71] and to be present in the axons of differentiated neurons in adults and during the growth of postmitotic neurites in the CNS [72,73]. Numb has also been detected in terminally differentiated neurons in the adult retina, suggesting a role after its exit from the cell cycle [49]. Numb and Msi1 are probably present in the

Figure 9. Msi-1 protein production in non-retinal tissues. High magnification of the cornea shows intense immunolabeling of the corneal epithelium (CEp; A), the corneal keratinocytes (A and B), and the corneal endothelial cells (CEn; C). D and E: Msi1 was observed in the limbal cells including the stem and progenitor cells. F: Msi1 immunoreactivity was also detected in the two pigmented layers of the iris and in iris stromal melanocytes and iris endothelial cells. G: Msi1 immunoreactivity was also detected in the pigmented ciliary epithelium (PCE) and nonpigmented ciliary epithelium (NPCE) of the ciliary body. H: Msi1 was observed in the lens epithelium (Le), transitional zone (Tz), and lens fiber (Lf). I: Significant immunoreactivity was observed in the retinal pigment epithelium (RPE) and the inner segment (IS).
same cell types in the adult retina. Double labeling experiments are required to demonstrate this hypothesis conclusively. Numb negatively regulates Notch signaling, and Notch1 is present in mature neurons in adult mouse and the human brain and in postmitotic neurons in vitro, suggesting that this protein may be involved in the physiology of mature neurons [74]. Thus, the entire pathway involving Notch, Numb, and Msi1 appears to function in mature neurons of the CNS including the retinal neurons. Finally, Msi1 has been shown to mediate the effects of the thyroid hormone on the maturation of tau mRNA [75]. The tau gene encodes a microtubule-associated protein that is important for the stabilization and organization of axonal microtubules therefore, crucial for neuronal morphology and polarity, neurite outgrowth, and axonal transport [76].

Msi1 has not previously been detected in adult brain neurons. The difference in Msi1 protein levels in the brain and retina suggested that Msi1 might be present in the central nervous system RNA granules, which are diverse and known to contain various RNA-binding proteins. The RBPs content of these RNA granules may vary from one region of the CNS to another in terms of both the nature and level of these molecules. These RBPs may bind different subsets of mRNA subpopulations. As highlighted above, Msi1 was found in the whole adult mouse eye and retina together with at least two other specific RBPs such as Stau2 and Pum2. These findings suggest that these RBPs may interact in identical or different retinal functions and in different or within the same RNA granules.

Our observations suggest that Msi1 functions are not restricted to the asymmetric division of early progenitor cells. Instead, this protein seems also to be involved in the cell cycle progression and differentiation of various cell types during retinal development and may also be involved in adult neuron function and physiology.

**Msi1 production in extra-retinal compartments:** Msi1 has been recognized as a possible intrinsic marker of several types of stem cells [48]. A recent study reported that multipotent neuroepithelial cells (NEP) express Msi1 and nestin [77]. However, the expression of Msi1 by neural crest stem cells and the ocular structures derived from them has not previously been investigated. Neural crest cells make a particularly important contribution to the anterior segment of the eye in terms of its optic physiology and histological complexity. In their seminal study, Johnston et al. [78] showed interspecific combinations involving either the cephalic mesoderm or the neural crest, demonstrating the extensive participation of mesodermal cells at this level.

**Msi1 production in the limbus:** The limbus links the margin of the scleral region to the irido-corneal angle and thus receives large numbers of neural crest cells [79]. Limbal basal epithelial cells, which maintain corneal epithelium homeostasis (for review see [80]), are not homogeneous. They consist of diverse populations of stem cells [80]. No single specific molecular marker for identifying limbal stem cells has yet been identified. We show here that the mouse limbal cells including the stem and progenitor cells of the limbus are strongly immunolabeled for Msi1. We therefore suggest that Msi1 may be an intrinsic marker of limbal stem cells.

**Msi1 production in the cornea:** The corneal epithelium and endothelium are formed from the first wave of mesenchymal cells whereas a second wave of neural crest-derived cells form the corneal stroma. These cells finally differentiate into keratocytes, which are actively involved in maintaining a transparent adult cornea.

Our results demonstrate that all layers of the adult corneal epithelium are immunoreactive for Msi1. The intense labeling of the corneal basal epithelium may reflect the involvement of Msi1 in normal corneal epithelium renewal and after epithelial injury. Indeed, various studies have demonstrated that Msi1 is produced by epithelial stem cells in the intestine [24,81] and the mammary gland [82]. These data are consistent with Msi1 being a candidate marker of adult basal progenitor cells of the corneal epithelium.

The adult cells constituting the corneal endothelium and the stromal keratocytes display intense immunostaining for Msi1. These two different types of adult corneal cells are derived from the embryonic neural crest mesenchymal stem cells. A recent study identified multipotent neural crest-derived stem cells in the adult mouse cornea. These cells were amplified in vitro; expressed the stem cell markers nestin, Notch1, Msi1, and ABCG2; and differentiated into adipocytes, chondrocytes, and neural cells [83]. A possible role for Msi1 in the transparency and optical properties of the cornea cannot be excluded as this protein is produced in keratocytes and in the corneal endothelium both of which are of essential importance in corneal transparency [84].

**Msi1 production in the lens:** The lens grows by means of epithelial cell proliferation and the differentiation of the progeny into secondary fibers at the lens equator. This process involves a major reorganization of the cytoskeleton with the elongation of cells into millimeter-long fibers [85]. The pattern of growth of the lens ensures that its polarity is maintained as secondary fibers are added to the fiber mass throughout the animal’s life. This is important for maintenance of the ordered cellular architecture that contributes to the physiological properties of the lens [86]. The terminally differentiated lens fiber cells lose their organelles, making it possible for the lens to become transparent [87]. We found that Msi1 was expressed from the embryonic stages to the postnatal and adult stages, suggesting the possible involvement of Msi1 in lens fiber cell proliferation, differentiation, and the maintenance of lens polarity and transparency.

**Msi1 production in the ciliary body, iris, and retinal pigmented epithelium:** During mammalian eye development, neural crest-derived cells form the ciliary body (CB) and the iris including their pigmented cells. We show here Msi1 immunoreactivity in diverse cells composing the ciliary body, the iris, and the entire RPE at the various postnatal stages studied and in adult mice. Retinal stem/progenitor cells have been identified in the CMZ in postnatal chickens [34,37,38] and in mammalian eyes during embryonic development [88]. In chickens, these populations of stem/progenitor cells can differentiate into retina-specific cell types [38]. In adult rodents, cells
located in the pigmented ciliary margin, corresponding topologically to the CMZ, have stem cell characteristics when cultured in vitro [37,38]. As Msi1 has been shown to be a marker of stem cells in different organs and in different compartments of the same organ, we suggest that Msi1 might be a marker of stem/progenitor cells located in the CB.

Msi1 expression was detected in the melanocytes of the adult ciliary body, in the melanocytes of the adult stromal iris, and in the two pigmented layers of the iris. Several teams have considered the pigmented layers of the iris to be a potential source of cells for transplantation experiments for testing potential neuroprotective effects or for the transdifferentiation of these cells into retinal cells in animal models of retinal degeneration [89,90].

Recent studies have reported that adult mammalian RPE cells have certain neural progenitor properties but cannot transdifferentiate into retina-specific neurons [91,92]. We detected Msi1 protein at all postnatal stages studied and in adult animals. This suggests that Msi1 may express a residual potential stem-cell or progenitor state in RPE cells. This potential may be reactivated in certain circumstances, after appropriate stimulation.

This distribution within different ocular compartments at various stages of ocular development and in the adult eye suggests that this regulator of posttranscriptional events may play an important role at multiple steps in eye development and in the maintenance and functions of most of the adult eye compartments including the neural retina and the RPE. Little is known about the mechanism controlling Msi1 gene expression. One recent study suggested that Msi1 may be subject to posttranscriptional control by ELAV RNA-binding proteins during the transition from proliferation to the neural differentiation of stem/progenitor cells [93]. The Msi1 sequence has also been reported to contain many Tcf/Lef binding sequences (Wnt signal response sequence) and Sox binding sequences in the regulatory regions conserved between species [48]. Wnt signaling and Sox family transcription factors have been found to play important roles in the induction and maintenance of different types of stem cells including intestinal, lens, corneal, mammary, hematopoietic, and certain types of neural stem/progenitor cells [94-97]. However, the genuine biological activity of the Tcf/Lef binding sequences (Wnt signal response sequence) and Sox binding sequences has not yet been demonstrated in any potential ocular target cell or tissue. It has been suggested that Msi1 production is induced by Wnt signaling and the action of Sox family transcription factors in turn activate Notch signaling by repressing the translation of Numb mRNA. All these possible interactions might lead to crosstalks between signaling pathways involving the self-renewal of stem cells [48]. A major question remains without a clear answer: Is Msi1 only a translational repressor playing diverse roles in distinct biological contexts or is it exclusively a stem/progenitor cell marker? On one hand, the expression of Msi1 in several types of adult differentiated cells supports the hypothesis of Msi1 fulfilling a general role of an RNA-binding protein acting as a translational repressor in several cell types. On the other hand, neural stem cells (NSCs) and progenitor cells (PCs) express a group of selective neural stem/progenitor cell marker molecules, the RNA-binding protein Msi1, the intermediate filament Nestin, and the transcription factor Sox (SRYlike HMG box)-family molecules [48]. The reconciliation of these apparently opposite roles of the Musashi-1 RNA binding protein might be provided by the study of Msi1 tissue specific conditional knockout mice. The different roles of the RNA-binding-protein Msi1 during development and adulthood are reminiscent of the different roles fulfilled by other major developmental markers of the eye such as the DNA-binding proteins, Pax6 and Chx10, which play a very different role in the cells where they are expressed during development and adulthood [98-100].

In summary, we have demonstrated for the first time that the RNA-binding protein Msi1 is produced in the mouse retina, ciliary body, iris, retinal pigment epithelium, lens, cornea, and limbus during development and in adulthood. Our results strongly suggest that Msi1 plays more diverse roles than anticipated in the developing mouse eye and in the adult mouse eye. Further studies are required to elucidate the functions of Msi1 in eye stem/progenitor cells as well as in many adult differentiated ocular cells in normal and pathologic conditions.

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