MRAS GTPase is a novel stemness marker that impacts mouse embryonic stem cell plasticity and *Xenopus* embryonic cell fate

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SUMMARY

Pluripotent mouse embryonic stem cells (mESCs), maintained in the presence of the leukemia inhibitory factor (LIF) cytokine, provide a powerful model with which to study pluripotency and differentiation programs. Extensive microarray studies on cultured cells have led to the identification of three LIF signatures. Here we focus on muscle ras oncogene homolog (MRAS), which is a small GTPase of the Ras family encoded within the *Pluri* gene cluster. To characterise the effects of *Mras* on cell pluripotency and differentiation, we used gain- and loss-of-function strategies in mESCs and in the *Xenopus laevis* embryo, in which *Mras* gene structure and protein sequence are conserved. We show that persistent knockdown of *Mras* in mESCs reduces expression of specific master genes and that MRAS plays a crucial role in the downregulation of OCT4 and NANOG protein levels upon differentiation. In *Xenopus*, we demonstrate the potential of *Mras* to modulate cell fate at early steps of development and during neurogenesis. Overexpression of *Mras* allows gastrula cells to retain responsiveness to fibroblast growth factor (FGF) and activin. Collectively, these results highlight novel conserved and pleiotropic effects of MRAS in stem cells and early steps of development.

KEY WORDS: LIF, *Mras*, mES cells, Neurogenesis, Pluripotency, Mouse, *Xenopus*

INTRODUCTION

Studies of stemness status in mouse embryonic stem cells (mESCs) through transcriptomic, proteomic and drug screenings have enabled the identification of many gene families potentially involved in the maintenance of cell pluripotency, among which are the regulators of Ras family (Chen et al., 2006; Ivanova et al., 2006; Wray et al., 2010). Understanding the function of newly discovered genes involved in the control of the pluripotency/differentiation balance in stem cells requires the use of complementary models to address cell biology and developmental questions in fast and efficient ways. In this report, we have characterised the effects of a novel regulated GTPase, muscle ras oncogene homologue (MRAS; also known as muscle and microspikes RAS), in mESCs and in *Xenopus laevis*. The convenient and efficient manipulation of gene expression in *Xenopus* embryos, along with studies performed in the mESC model, allow information concerning the basic conserved function of genes during evolution to be examined.

mESCs are maintained in a pluripotent state in the presence of the leukemia inhibitory factor (LIF) cytokine, which induces the JAK1/STAT3/SOC3 pathway that forms part of the pluripotency circuitry along with Wnt and bone morphogenetic protein 4 (BMP4) secreted factors, and the master genes such as *Oct4* (*Pou5f1*), *Nanog* and *Sox2* (Boeuf et al., 1997; Duval et al., 2000; Niwa, 2007; Chambers and Tomlinson, 2009; Trouillas et al., 2009a). Extensive microarray studies, performed in mESCs grown under pluripotent conditions (with LIF) or induced to differentiate (24 hours or 48 hours without LIF) and restimulated for a short period of time by LIF, have led to the identification of three LIF signatures (*Pluri*, *Spe-Lifind* and *Pleio-Lifind*) (Mathieu et al., 2012; Trouillas et al., 2009a; Trouillas et al., 2009b). An interesting feature of genes from the *Pluri* cluster is their early downregulation upon LIF withdrawal as compared with the master genes (Schulz et al., 2009; Trouillas et al., 2009b).

*Mras* is a member of the *R-ras* subfamily of the large *Ras* family and encodes a small GTPase (Díez et al., 2011). In mESCs, *Mras* is in the *Pluri* gene cluster, with an expression profile similar to that of *Esrrb* and *Tcl1* (Ivanova et al., 2006; Trouillas et al., 2009b). *Mras* expression is under the control of the KLF5 transcription factor (Parisi and Russo, 2011; Parisi et al., 2010), with elevated transcripts in specific brain areas, heart and the developing bones. The protein is concentrated on plasma membrane-associated structures (Kimmelman et al., 1997; Kimmelman et al., 2002; Matsumoto et al., 1997; Watanabe-Takano et al., 2010). MRAS expression and its GTPase activity are stimulated by nerve growth factor (NGF) in pheochromocytoma PC12 cells, in which it triggers ERK-dependent neuronal differentiation (Kimmelman et al., 2002; Sun et al., 2006). MRAS expression is also stimulated by the BMP2 signalling pathway in myoblast C2C12 cells, in which it induces osteoblast differentiation under the control of the p38 and JNK kinases (Watanabe-Takano et al., 2010). Activated forms of MRAS, such as the MRAS-G22V mutant which is constitutively in the GTP-bound form, mimic inducers (NGF or BMP2) and trigger neuronal or osteoblast differentiation in PC12 or C2C12 models, respectively (Kimmelman et al., 2002; Sun et al., 2006; Watanabe-Takano et al., 2010), induce formation of peripheral microspikes in fibroblasts and participate in the reorganisation of the actin filament cytoskeleton. *Mras* as a regulator of actin microspikes has been recently reviewed (Barnard et al., 2011).

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cytoskeleton (Matsumoto et al., 1997). In addition, these activated forms are oncogenic in murine mammary epithelial cell lines (Ward et al., 2004). MRAS function is potentially regulated by plexin B1, a dual-function GTPase-activating protein (GAP) for RRAS and MRAS, which remodels axon and dendrite morphology, respectively (Saito et al., 2009).

There is a single Mras gene from nematodes to mammals and in the ascidian embryo the orthologue has a role in neural differentiation, suggesting an evolutionarily conserved function (Keduka et al., 2009). The *Xenopus* embryo has contributed substantially to the deciphering of the molecular mechanisms that control embryo patterning, which relies on signalling pathways that are highly conserved between mammals and amphibians (Harland and Grainger, 2011; Heasman, 2006). LIF and its receptors (gp130 and gp190), along with the Pluri genes (such as Esrrb, Sod2, Cd9, Fzd5, Susd2 and Smad3), are conserved in *Xenopus laevis* (our unpublished data). In addition, cells from the animal part of the *Xenopus* blastula embryo (animal cap cells) are similar to mammalian ESCs. They are pluripotent and able to differentiate toward the lineage-specific cell types of the three germ layers (Lan et al., 2009; Okabayashi and Asashima, 2003). Moreover, the molecular mechanisms that govern self-renewal mediated by Oct4 are conserved between mammals and *Xenopus* (Morrison and Brickman, 2006; Abu-Remaileh et al., 2010). This makes *Xenopus* a suitable model in which the basic conserved functions of these genes can be studied during early embryogenesis and organogenesis, two processes not easily amenable in the mouse embryo. For instance, our recent work using a combined approach of mESCs and *Xenopus* embryonic cells has demonstrated a conserved function for a histone methyltransferase adaptor protein in smooth muscle cell differentiation (Gan et al., 2011).

In this study, we have carried out a functional analysis of *Mras* in mESC and *Xenopus* models, leading to the characterisation of *Mras* as a novel stemness marker that impacts on pluripotent cells and on neurogenesis.

**MATERIALS AND METHODS**

**Ethics statement**

This study was carried out in accordance with the European Community Guide for Care and Use of Laboratory Animals and approved by the Comité d’ethique en expérimentation de Bordeaux, No. 33011005-A.

**Cell culture**

CGR8, E14Tg2aWT and E14Tg2a GeneTrap Mras (feeder-free) ESC lines were grown as described (Trouillas et al., 2009b). The E14Tg2a GeneTrap Mras cell line (E14Dmras) was purchased from the Mutant Mouse Regional Resource Center (MMRRC) under the strain name BayGenomics ESC line CSH312, stock number 014915-UCD. This cell line contains in exon 2 of *Mras* an insertion of the β-geo fusion cDNA in one allele. A half dose of *Mras* expression was validated with two sets of primers (sequences are listed in supplementary material Table S1). The E14Tg2aWT (E14WT) control cell line was purchased from ATCC.

**Heat map analysis**

Heat map analysis was performed using the FunGenES website interface (Schulz et al., 2009; Trouillas et al., 2009b).

**Cell lysates and western blots**

Plated ESCs were rinsed twice at room temperature with PBS and lysed directly in mild RIPA buffer (Trouillas et al., 2009b). Total protein (100 μg) was loaded on 10% SDS-PAGE gels, transferred to nitrocellulose membranes and incubated with anti-OCR4 (Abcam, 18976; 1:500), anti-NANOG (Abcam, 21603; 1:1000) and anti-ERK2 (Santa Cruz Biotech, sc-154; 1:5000) antibodies.

**Flow cytometry**

Plated cells were washed once at room temperature with PBS, trypsinised and counted. Cells were fixed for 20 minutes in 4% paraformaldehyde, permeabilised with Pern/Wash Buffer (BD Biosciences) for 10 minutes and labelled with 20 μl antibody for 1 hour with the Mouse Pluripotent Stem Cell Transcription Factor Analysis Kit (BD Biosciences). After two washes with the Wash Buffer for 10 minutes, cells were processed on a FACSCanto II flow cytometer (BD Biosciences). The percentage of positive cells and the mean fluorescence intensity (MFI) were obtained with DIVA software (BD Biosciences).

**RT-PCR and RT-qPCR**

Total RNA from adherent ESCs was prepared with Trizol reagent as described (Trouillas et al., 2009b). The reverse transcription reaction products were used for qPCR with specific primer sets and relative expression was calculated using *Hprt* (CGR8 cell line) or *Erk2* (Mgk1) (E14 cell lines) as reference genes. RNA from *Xenopus* embryo and explants was prepared and analysed according to standard protocols (Sive et al., 2000; Nayé et al., 2007). Primers are listed in supplementary material Table S1.

**Construction of expression vectors and establishment of stable clones**

Expression vector construction

RT-PCR-amplified fragments of *Mus musculus Mras* (NM_008624) were inserted in fusion with *EGFP* cDNA in the *Nhel/Xhol* site of the pcXN2 vector (Niva et al., 1991). A 1.2 kb *Xenopus laevis Mras* cDNA was cloned by RT-PCR from stage 20 embryo RNA into the *EcoRI/Xhol* sites of the pcS2 vector (a gift from R. Rupp) using gene-specific primers: forward, 5′-cgcgcattcATGGCAACGAGTCTGTTCTGAG-3′; reverse, 5′-cgcgtagcTCACAATACGACACTGAGGCT3′.

Mouse *Mras* was cloned from pCAG-EGFP-MRAS into pcS2 by digestion with *Nhel* (end filled)/Xhol and ligation into the *EcoRI* (end filled)/Xhol sites of pcS2. MRAS G22V and MRAS S27N encoding sequences were cloned from the pEF-BOS-MyoCMras constructs (Sun et al., 2006) into the BamHI and Xhol sites of pcS2. Plasmid constructs were checked by DNA sequencing.

**Derivation of mESC stable clones**

To establish ESC clones expressing EGFP or EGFP-MRAS, CGR8 cells maintained in LIF-containing medium were transfected using Lipofectamine (Invitrogen) with 0.5-2 µg pcXN2-derived construct per 6-cm Petri dish. After 2 weeks (for EGFP) and up to 4 weeks (for EGFP-MRAS) of selection in the presence of 300 µg/ml G418, double-positive clones (Neo8 and EGFP) were picked and grown in the presence of G418. Images were captured on a Zeiss Axiovert 200 microscope with an AxioCam HR camera and Axiovision 3.0 and Axioviewer 3.0 software.

**Embryological methods**

*Xenopus laevis* embryos were obtained by standard procedures (Sive et al., 2000) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Animal cap explants were dissected from stage 8 or stage 11 embryos, treated with FGFR or activin (R&D Systems) and cultured until the control embryos reached the appropriate stage before RT-PCR analysis. For microinjection experiments, capped mRNAs were synthesised using the mMessenger mMachine SP6 Kit (Ambion). Injections were performed into one blastomere at the 2-cell stage, with 250 pg *lacZ* mRNA as lineage tracer. For knockdown experiments, 20-40 ng morpholinos (MOs) (Gene Tools; supplementary material Table S2) were injected into 2-cell stage embryos. In the animal cap assay, 500 pg *Mras* mRNA and 50 pg *noggin* mRNA were injected into the animal pole of the 2-cell stage embryo in both blastomeres.

**Whole-mount in situ hybridisation and immunohistochemistry**

In *situ* hybridisation and immunohistochemistry were performed according to standard protocols (Sive et al., 2000). For serial sections, embryos post-fixed in MEMFA (0.1 M MOPS, 2 mM EGTA, 1 mM MgSO4 and 3.7% formaldehyde) were embedded in Paraplast (Sigma) and cut on a microtome. The 12/101 antibody (Developmental Studies Hybridoma Bank) was used at 1:2 dilution.
In vitro translation

In vitro transcribed mRNAs (500 ng) were translated in rabbit reticulocyte lysate (Promega) and the reaction products were analysed by 12% SDS-PAGE followed by autoradiography.

Sequence analysis

The *Mus musculus* MRAS protein sequence (NM_008624) was BLASTed in the SWISS-PROT database and the sequences of various organisms were retrieved and aligned using the MultiAlin tool (Corpet, 1988). Phylogenetic analyses were performed with the following sequences: *Xenopus laevis* (Xl), NP_001085506; *Homo sapiens* (Hs), NM_012219; *Canis familiaris* (Cf), XP_534277; *Mus musculus* (Mm), NM_008624; *Rattus norvegicus* (Rn), NM_012981; *Gallus gallus* (Gg), NM_204489; *Danio rerio* (Dr), 003201047. Genes structure and intron size comparisons were retrieved from Ensembl.

RESULTS

Mras, a new marker of stemness

Extensive microarray analyses performed with mESCs grown under different culture conditions (pluripotency or differentiation induction) have enabled the identification of novel markers of pluripotent mESCs and the characterisation of a set of *Pluri* genes that are highly expressed in undifferentiated mESCs (Schulz et al., 2009; Trouillas et al., 2009a; Trouillas et al., 2009b). Here, we focus on the expression profiles of *Ras* family members. Probe set numbers of this gene family and related members (including the *Ras-Gap* and *Ras-GeF* genes, 273 probe sets) were retrieved from the Affymetrix 420.2 microarray and their expression profiles analysed in pluripotent cells and after LIF withdrawal from 1 to 10 days. A heat map representation of a selection of genes (46 probe sets) is presented (Fig. 1A).

This analysis showed heterogeneous expression profiles of *Ras* members, and a particular profile for *Mras*. Indeed it is the only member that is restricted in its expression to undifferentiated ESCs, with a drastic reduction of expression as early as 1 day after LIF withdrawal. We confirmed, using RT-qPCR analysis, that *Mras*, but not *Hras*, *Kras*, *Rras* or *Eras*, is regulated by LIF (Fig. 1B). We also showed that *Mras* expression is under LIF control only in undifferentiated cells. Indeed, *Mras* expression is not induced by short LIF treatment in committed (24 hours minus LIF) or differentiated (48 hours minus LIF) cells, in contrast to the *Lifind* genes, which respond to LIF in ESC-derived committed and differentiated cells and in other mature LIF-responsive cell types (Trouillas et al., 2009a; Trouillas et al., 2009b; Mathieu et al., 2012). These results revealed that *Mras* constitutes a new marker of stemness.

Persistent downregulation of *Mras* leads to a decrease in the expression of core pluripotency markers

To determine whether *Mras* controls the fate of pluripotent cells, the expression of the core pluripotent genes (*Oct4*, * Sox2* and *Nanog*) and a selection of *Pluri* or differentiation markers was examined in cells in which *Mras* expression was knocked down. We used a clonal heterozygous GeneTrap *Mras* ESC line (E14Dmras), which harbours an insertion of a β-geo cassette in exon 2 (see Materials and methods). *Mras* expression was decreased by 50% in this cell line when compared with the parental E14WT mESC line (Fig. 2A). This mutated cell line, despite being alkaline phosphatase positive in the presence of LIF, displays a reduced self-renewal efficiency (7%, versus 35% for E14WT; supplementary material Fig. S1A).

To further characterise the deficit caused by decreased expression of *Mras*, E14WT and E14Dmras were grown in the presence or absence of LIF for 4 days. Whereas there was no
change in the expression of Ras family members (Fig. 2A), significant downregulation in the expression of the Pluri genes Ceacam1 and Esrrb and the master genes Oct4 and to a lesser extent Nanog was observed in the mutated cell line, with a concomitant decrease in OCT4 and to a lesser extent NANOG protein (Fig. 2B,D). Under these conditions, no significant differences in the expression of the differentiation markers tested were detected between WT and Dmras cells (Fig. 2C). However, we have reproducibly observed that the formation of embryoid bodies (EBs) is slower and that they are smaller in Dmras versus WT lines. Furthermore, analysis of differentiation markers at two time points in differentiation (8-day EBs and 13 days after EB plating) shows that there is a decrease in endodermal (Sox17) and mesodermal [brachyury (T)] but not neuronal [Tuj1 (Tubb3) and Map2] expression markers in Dmras versus E14WT only in plated differentiated cells (data not shown).

These results suggest that the decrease in Mras expression, which affects the proper balance in the expression of pluripotent genes, leads to an unpoised state of the cells, which have lost, in part, their potential to respond to differentiation cues.

**Overexpression of MRAS leads to different cellular phenotypes**

MRAS overexpression or its activation was previously shown to induce differentiation in various cell models (Kimmelman et al., 2002; Sun et al., 2006; Watanabe-Takano et al., 2010). We tested the effect of overexpression of MRAS in mESCs grown with or without LIF. MRAS protein expression was detected in 30-50% of the transfected cells (data not shown) the day after transfection, with an average of 10-fold overexpression of the transfected gene in transient transfection versus endogenous Mras expression (Fig. 3A,B, Fig. 4A).

Cells transfected with the EGFP-MRAS fusion protein display specific morphological changes compared with EGFP control cells. Formation of membrane microspikes was observed 3 days after transfection and an increase in the proportion of differentiated cells was observed after 8 days of selection (Fig. 3A). Although differentiated cells could be maintained for more than a month without apparent proliferation, they died after the first passage following trypsin treatment. In addition, after 4 weeks in selective medium with LIF, stable EGFP-MRAS clones, with a slow growth rate compared with the EGFP clones, were recovered and amplified (Fig. 3B, Fig. 4A; supplementary material Fig. S1B,C).

Our data suggest that Mras, like Oct4 and Sox2, behaves as a rheostat gene that displays different morphological effects depending upon its level of expression, which is heterogeneous following transfection (Kopp et al., 2008; Niwa et al., 2000).

**Stable clones overexpressing MRAS have sustained expression of OCT4 and NANOG when grown without LIF**

To determine whether overexpression of MRAS impacts the core pluripotent complex, clones expressing EGFP or EGFP-MRAS were grown with or without LIF for 7 days. Sustained expression of OCT4 was observed in Mras-overexpressing clones grown without LIF (Fig. 4B; supplementary material Fig. S2). In addition, protein expression was analysed by flow cytometry on the EGFP-positive cell fractions. A representative analysis of four clones (EGFP-MRAS-A to -D) displays sustained expression not only of OCT4...
but also of NANOG upon LIF withdrawal (Fig. 4C). This finding could indicate that cell differentiation has been delayed upon MRAS overexpression. The ERK and PI3K signalling pathways, which are involved in cell differentiation and self-renewal in mESCs, respectively, have been shown to be activated by MRAS in different situations (Kimmelman et al., 2000; Kimmelman et al., 2002). However, we did not observe a constitutive induction of these pathways when \textit{Mras} is overexpressed (supplementary material Fig. S3).

Since MRAS-overexpressing clones grew slowly and were difficult to maintain in culture, it was not possible to use them for a more in-depth analysis. Therefore, to explore the functions of \textit{Mras} in cell fate decision we turned to the \textit{Xenopus} model, which has been fruitful for establishing the functional conservation of pluripotent genes.

\textbf{Mras gene structure and protein sequence are conserved among vertebrates}

\textit{Xenopus} MRAS protein shows 84% and 92% identity with the zebrafish and human orthologues, respectively (Fig. 5A). This identity is in agreement with the phylogenetic analysis of the \textit{Ras} family, which indicates that a single \textit{Mras} orthologue is present in the genome from tunicates to mammals (Keduka et al., 2009). \textit{Mras} gene structure is remarkably conserved among vertebrates and is composed of five exons, interrupted by four introns with identical splice site junctions, having a conserved coding capacity except for the first exon of the zebrafish gene (Fig. 5B). The intronic regions of mammalian \textit{Mras} genes have a very long first intron compared with those of the chicken, \textit{Xenopus} and zebrafish orthologues (Fig. 5C).

\textbf{Xenopus Mras expression is maternal and zygotic and restricted to the adult brain and ovary}

Semi-quantitative RT-PCR analysis indicates that \textit{Mras} is expressed in unfertilised \textit{Xenopus} eggs and at all embryonic stages analysed, but that the maternal transcript level decreases after blastula stage (stage 9) and zygotic expression starts at late neurula stage (stage 17) (Fig. 6A). In the blastula embryo, \textit{Mras} mRNA is found throughout the embryo, with no difference between animal and vegetal poles, whereas in the gastrula embryo (stage 10.5) it is mainly found at the animal pole (Fig. 6B). In adult tissues, \textit{Mras} is mainly expressed in the brain (as in mammals) and ovary (Fig. 6C).

\textbf{Rohon-Beard cells and trigeminal neurons are the major sites of expression of Mras in the \textit{Xenopus} embryo}

We did not detect, through whole-mount \textit{in situ} hybridisation, any localised \textit{Mras} expression in the early \textit{Xenopus} embryo before stage 24, when a faint labelling was observed in a string of cells along the nerve cord corresponding to Rohon-Beard (RB) cells, which are primary sensory neurons (Roberts, 2000) (Fig. 7A,B). Later in development, labelling also appears in the trigeminal neurons and epiphysis and is clearly detected between somites on each side of the neural tube (Fig. 7C-H).

\textbf{Mras regulates neuronal differentiation}

\textit{Mras} overexpression induces the neuronal differentiation of PC12 mammalian cells (Kimmelman et al., 2002; Sun et al., 2006). Moreover, microarray data analysis indicates that \textit{Mras} is re-expressed in ESC-derived neuronal cells (Abranches et al., 2009;
Schulz et al., 2009) (supplementary material Fig. S4). Because Mras is expressed in RB cells of the Xenopus embryo, we investigated the effects of its loss-of-function on neuronal differentiation during development through the use of an antisense morpholino oligonucleotide strategy. A morpholino directed against Mras sequence (MO Mras) and a mismatch morpholino (MO mismatch) were tested for their ability to block Xenopus Mras translation. Only MO Mras reduced translation of Mras mRNA (supplementary material Fig. S5). Morpholinos were injected into one cell of 2-cell stage embryos and morphant

**Fig. 4.** Overexpression of MRAS in the absence of LIF leads to sustained expression of OCT4 and NANOG. (A) Relative expression level of Mras as determined by RT-qPCR. The endogenous level of Mras in mESCs + LIF or without LIF for 24 hours or transfected with 1 μg pCAG-EGFP (EGFP) (lanes 1–4) is compared with the level of overexpressed Mras after transfection with pCAG-EGFP-MRAS (lanes 5, 6) the day after transfection. The expression of Mras in stable clones selected for a month in G418 in complete medium with LIF is shown for EGFP clones (lanes 7–9) and EGFP-MRAS clones (lanes 10–12). The mean fluorescence intensity (MFI) of transfected cells is, on average, 15,000 the day after transfection. (B) OCT4 and ERK2 western blot analysis of cell lysates from stable clones expressing EGFP or EGFP-MRAS with and without LIF. (C) FACS analysis of the percentage of cells expressing OCT4, NANOG or SOX2 among WT cells (the mean of three experiments is shown with s.d.) or representative individual clones expressing EGFP or EGFP-MRAS in the presence or absence of LIF for 7 days. The mean MFI is 2894 for clone A, 3000 for clone B, 3000 for clone C and 3822 for clone D, with a background autofluorescence of 100.

**Fig. 5.** Conservation of vertebrate Mras at both protein and genomic levels. (A) MRAS protein sequence comparison between dog (Cf, Canis familiaris), human (Hs, Homo sapiens), mouse (Mm, Mus musculus), rat (Rn, Rattus norvegicus), chicken (Gg, Gallus gallus), Xenopus (Xl, Xenopus laevis) and zebrafish (Dr, Danio rerio). Identical amino acids are black, highly conserved in blue and neutral in red. (B) Vertebrate Mras gene structure with exons represented by black boxes, introns (I-IV) by a thin line and untranslated regions by white boxes. The length of the coding sequence (in nucleotides) is indicated for each exon. The type of splice junction is indicated for each intron. (C) Intron size (in nucleotides) comparison between the different Mras genes.
Embryos were analysed for the expression of the neuronal marker N-tubulin, which marks zones of primary neurogenesis in the embryo. In the stage 15 Xenopus embryo, N-tubulin is expressed in three stripes of cells that correspond to primary motor, interneuron stripe (75%, n=126) and of the trigeminal neurons, with a milder effect on interneurons and almost no effect on motor neurons (Fig. 8).

The reduction in N-tubulin expression is specific as neither MO mismatch nor MO standard control (GeneTools) impaired its expression and, moreover, the injection of a Mras mRNA that is insensitive to MO Mras can rescue N-tubulin expression (Fig. 8). Additional markers of neuronal differentiation were also investigated: Elrc, Runx1, Pak3 and Islet1 (Perron et al., 1999; Souopgui et al., 2002; Carruthers et al., 2003; Brade et al., 2007). The expression of Elrc (72%, n=77), Pak3 (71%, n=42) and Islet1 (81%, n=32) but not of Runx1 (3%, n=86) is reduced in MO Mras morphants (Fig. 8). Together, these experiments indicate that Mras might function upstream of Elrc, Pak3 or Islet1 in the regulation of primary neuron formation but downstream or in parallel of Runx1 in the generation of RB sensory neurons.

**Mras acts at a late step of neurogenesis and is required for neuronal differentiation**

The early pan-neural marker Sox2 is essential for neuroectoderm formation, while Neurogenin is the earliest proneural gene expressed within the neural plate (Kishi et al., 2000; Ma et al., 1996). Mras depletion in the Xenopus embryo did not affect the expression of either marker (0%, n=65 for Sox2 and n=54 for Neurogenin), indicating a late role for Mras in neuronal differentiation (Fig. 9).

A constitutively active mutant of Mras (G22V) has been shown to induce neuronal differentiation of PC12 cells, whereas a dominant-negative form of the protein (S27N) impaired neuritogenesis (Sun et al., 2006). We tested whether these two forms of Mras had an impact on neuronal differentiation. Whereas the constitutively active form of MRAS induced increased expression of N-tubulin (69%, n=86), the dominant-negative form decreased N-tubulin expression (80%, n=25). Together, these data indicate that Mras is acting at a late phase of neuronal differentiation and that its activation is a prerequisite to its function during differentiation.

**MRAS maintains the competence of gastrula embryo cells for FGF and activin induction and stimulates neural differentiation**

Embryonic cell patterning of the early Xenopus embryo relies on a limited number of signalling pathways that influence cell fate decisions that can be reproduced in pluripotent animal cap cells of the blastula embryo (Heasman, 2006). We found that FGF2 stimulates Mras expression in animal cap cells, whereas activin or BMP treatments have no significant effects (Fig. 10A). Knockdown
with MO Mras did not affect mesoderm induction mediated by FGF2 (Fig. 10B) or activin (data not shown), indicating that Mras is not essential for mesoderm induction.

The Xenopus animal cap assay has been successfully used to demonstrate the involvement of OCT4 and BRG1 in the maintenance of pluripotency (Hansis et al., 2004; Snir et al., 2006). Animal cap cells from the blastula embryo can be induced to form mesoderm when treated with FGF2 or activin, but gastrula stage embryos lose this property (Grimm and Gurdon, 2002; Snape et al., 1987). Animal cap cells from the gastrula stage embryo injected with Mras mRNA still express Xbra in response to FGF2 (Fig. 10C). Both Xenopus and mouse Mras showed this property (data not shown). Other marker genes (Cdx4, Pnp and Esr5) previously shown to be FGF targets (Branney et al., 2009) are also re-expressed in late animal cap cells upon Mras expression (Fig. 10C). Similarly, we tested the effect of Mras on the responsiveness of animal cap cells to activin. When Mras mRNA is overexpressed in the embryo, late animal cap cells can still respond to activin treatment and express Xbra, Chordin and Xnr1 (Fig. 10D). Together, these results indicate that Mras can maintain the mesodermal competence of pluripotent animal cap cells to FGF2 and activin. This property is not limited to mesoderm but can also apply to endoderm, as shown by the expression of the endoderm-specific gene Sox17 (Hudson et al., 1997) (Fig. 10D).

When considering the neurectoderm germ layer, the animal cap test cannot be used because both early and late animal cap cells respond to neurogenesis induction by the neural inducer noggin (Lamb et al., 1993). When Mras is overexpressed in animal caps with noggin, we observed a strong induction of the N-tubulin and Ncam genes, indicating a positive effect of Mras on neuronal differentiation (supplementary material Fig. S6). Together, these results support a conserved role of Mras in neurogenesis and describe a new function of this GTPase at the onset of gastrulation.

**DISCUSSION**

In this study, we have focused on Mras, which encodes a GTPase that is preferentially expressed in undifferentiated mESCs and re-expressed in ESC-derived neuronal cells. The impact of Mras was...
Mras in mESCs and Xenopus embryo

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Fig. 10. Mras can maintain the competence of Xenopus gastrula embryo cells for FGF and activin induction but is not required for mesoderm induction. RT-PCR analysis of gene expression in Xenopus animal caps cultured until the control embryo (lane E) reached stage 12.5. (A) Activin, BMP or FGF2 treatment followed by Mras and Xbra expression analysis. (B) Animal caps from Mras morphants (MO) or control embryos treated with 50 or 100 ng/ml FGF2 (FGF50 or FGF100) and analysed for Xbra expression. (C) Animal caps dissected at stage 8 (Early) or stage 11 (Late) from embryos injected with Mras or Brg1 mRNAs or uninjected embryo (Control) were treated with FGF2 (F) or activin (A) or left untreated (–) before expression analysis of Xbra, Cdx4, Pnp, Er5, Xnr, Chordin (Chd) or Sox17 (Sox). Odc was used as control, and a reaction was performed in the absence of reverse transcriptase (–RT).

evaluated in two models of pluripotent cells – mESCs and Xenopus blastula embryonic cells (animal cap) – and on the development of the Xenopus embryo.

The involvement of Ras GTPase members in the maintenance of cell pluripotency in mESCs was suggested by the fact that a chemical inhibitor of RasGAP, which maintains Ras members in a GTP-bound active conformation, allows the propagation of mESCs in an undifferentiated pluripotent state in the absence of LIF (Chen et al., 2006). In addition, a recent report has demonstrated the importance of the nuclear GTP-binding protein nucleostemin (Gnl3) for the self-renewal of mESCs by direct action on the G1 phase of the cell cycle (Qu and Bishop, 2012).

Functional approaches have demonstrated various roles for Ras members in mESCs: ERAS (ES cell-expressed ras) promotes ESC proliferation and the formation of teratomas (Takahashi et al., 2003); the activated KRAS mutant (G12V) alters the expression of pluripotent markers, leading to growth maintenance even with a low dose of LIF (Luo et al., 2007); and activated HRAS mutants induce differentiation toward trophoderm or extra-embryonic endoderm lineages (Yoshida-Koide et al., 2004; Lu et al., 2008). However, knockout mice failed to reveal any early embryonic function as almost all individual Ras knockouts are viable and fertile, except for Kras<sup>-/-</sup> mice, which display lethality at E12 (Ise et al., 2000; Koera et al., 1997; Nuñez Rodriguez et al., 2006; Umanoff et al., 1995). In this study, we have not observed a compensatory effect in Ras member expression in ESC lines expressing a half dose of Mras in the presence or absence of LIF. Further studies will be required to determine which genes, if any, might compensate for the absence of individual Ras members and to reconcile the lack of an embryonic phenotype in knockout mice with the crucial role played by some Ras members in the mESC model.

Our results identify a novel role for MRAS, which was thus far known for its involvement in differentiation processes (Kimmelman et al., 2002; Sun et al., 2006; Watanabe-Takano et al., 2010). We showed that downregulation of Mras expression in mESCs deconstructs the stemness program by decreasing the expression of Oct4 and of some Pluri genes, such as Ceacam1 and Esrrb, and diminishes the self-renewal capacity of the cells. However, the Dmras cell line and cells in which Mras has been repressed by shRNA (Parisi et al., 2010) maintain their undifferentiated morphology and are alkaline phosphatase positive, indicating that compensatory effects allow cells to grow and to be amplified in LIF-containing culture medium.

We also showed that overexpression of Mras leads to changes in cell morphology, with more giant and spiky differentiated cells observed within the first week of cell culture. However, we did not detect any MRAS-dependent induction of the ERK pathway in these early differentiated cells, which did not survive after the first passage in culture (data not shown; supplementary material Fig. S3). Stable clones overexpressing MRAS, which do eventually grow in selective medium, exhibit, in contrast to the wild-type clones, sustained expression of Oct4 and NANOG when cells are deprived of LIF for 7 days, with no effect on SOX2 expression. This indicates that MRAS activates a pathway that alters only the OCT4/NANOG subcomplex and probably not the SOX2/OCT4/NANOG complex. Oct4 is the paradigm of a rheostat effector gene, which modulates the stemness/differentiation balance depending on its level of expression (Niwa et al., 2000). In addition, an OCT4 dose-dependent switch from the Sox2 to Sox17 promoter triggers mesodermal differentiation (Blin et al., 2010; Stefanovic et al., 2009). Our data suggest that Mras behaves as a rheostat gene and that there is a link between MRAS and OCT4 expression. In cells deprived of LIF for a week, OCT4 mRNA expression diminishes whether or not Mras is overexpressed (our unpublished data). Therefore, we favour an MRAS-dependent post-translational stabilisation of OCT4 that could lead to sustained expression of the protein in Mras-overexpressing cells grown without LIF. Such post-translational modifications (phosphorylation or ubiquitylation) alter OCT4 stabilisation (Xu et al., 2009; Saxe et al., 2009). Interestingly, Mras along with Tcf1, another member of the Pluri cluster (Trouillas et al., 2009b), are direct targets of the KLF5 transcription factor, which is involved in the maintenance of cell pluripotency (Parisi et al., 2008; Parisi et al., 2010). MRAS could be a signalling relay of KLF5, acting both on gene transcription and the protein stabilisation machinery.

Because stable clones grew slowly, it was not possible to determine whether they remain pluripotent in the absence of LIF using teratoma formation. In addition, we observed a poor capacity of the E14Dmras cell line to form EBs in comparison to WT cells, and found that Brachyury (T) and Sox17 expression was decreased in comparison to the WT situation in late differentiated cells, indicating that a proper level of Mras is required for the cells to maintain their pluripotency and to differentiate efficiently.
Therefore, we took advantage of the complementary Xenopus model to address Mras functions in pluripotency and early development. We used both the in vivo approach and the model of pluripotent animal cap cells (Sasai et al., 2008). Mras is highly conserved among vertebrates from zebrafish to human at both the gene and protein levels, suggesting a selection pressure and conserved function. As in mESCs, there are clearly two phases of Mras expression in Xenopus, with a maternal expression that decreases between the blastula and early gastrula stages followed by a zygotic expression that increases in the early tadpole, a time when Mras mRNA is found in RB cells and trigeminal neurons. RB neurons are a transient population of primary sensory neurons that is observed in non-mammalian vertebrates, such as zebrafish and Xenopus, and is replaced in the late embryo by dorsal root ganglia (Holland, 2009; Olesnicky et al., 2010; Roberts, 2000). Strikingly, in the mouse embryo, Mras is also expressed in dorsal root ganglia (Keduka et al., 2009). We conclude that the amphibian and murine Mras genes share both strong sequence conservation and expression pattern (Kimmelman et al., 2000).

Loss-of-function of Mras in morphant embryos results in a consistent phenotype that is characterised by the suppression of neuronal differentiation as assessed by N-tubulin, Pak3, Elc or Islet1 expression. This is supported by experiments in which a dominant-negative form results in fewer neurons. By contrast, a constitutively active form of MRAS stimulates neuronal differentiation, expanding the N-tubulin expression domain (Fig. 9). Although Mras has been shown to induce neuronal differentiation, including neurite outgrowth in PC12 cells (Sun et al., 2006), our data constitute the first report of the involvement of Mras during vertebrate neurogenesis in vivo. We hypothesise that this property is not restricted to amphibians but is shared by mammalian species because the murine protein is able to rescue morphant embryos as efficiently as the amphibian protein. Mras acts at a late phase of neurogenesis, downstream of Sox2 and Neurogenin. Surprisingly, Runx1 expression is unchanged in Mras morphant embryos. Because Runx1 is expressed in RB progenitors and has been shown to be required for their specification independently of Neurogenin (Park et al., 2012), we hypothesise that Mras can act either downstream of Runx1 in a common pathway or independently of Runx1 in a parallel pathway.

In mESCs, a half dose of Mras leads to downregulation of Ceacam1, Esrrb, Oct4 and, to a lesser extent, Nanog, whereas its overexpression in LIF-deprived cells leads to sustained expression of OCT4 and NANOg, suggesting a role in pluripotency maintenance. We have demonstrated that gastrula cells that overexpress Mras can be induced to form mesoderm when treated with FGF2, suggesting that Mras can prolong their pluripotency, as shown for Brg1 (Smarca4) (Hansis et al., 2004). Mras is also able to maintain gastrula cell competence to form mesoderm, but also endoderm, under the control of activin. An evaluation of whether OCT4 expression is modulated in Mras morphant embryos was not possible owing to the lack of a Xenopus OCT4 antibody. However, we have not observed any variation in Oct4 expression at the mRNA level in Mras morphants (data not shown).

In conclusion, we have observed in both models two phases of Mras expression that could support two distinct roles. Mras expression in LIF-treated ESCs declines after LIF removal and is re-induced during neural differentiation. In Xenopus, the elevated Mras expression that is observed throughout the embryo until blastula stage could be required for the maintenance of pluripotency, whereas its localised expression in the presumptive neuroectoderm of the gastrula embryo supports its function in neurogenesis. Indeed, in pluripotent cells neuralised by noggin, Mras expression stimulates neuronal differentiation. Our work, along with the recent report on the function of the GTP-binding protein nucleostemin in the self-renewal of mESCs (Qu and Bishop, 2012), provide new tools and insights for characterising and understanding the links between GTP-binding proteins and stemness and the neurogenic regulators in different models such as mESCs and the Xenopus embryo. In addition, our study reinforces the use of complementary models for a better understanding of gene functions in vertebrates and demonstrates the involvement of Mras in cell pluripotency and neurogenesis.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
M.-E.M., C.F., C.S., F.S., X.G. and M.T. performed experiments and contributed to data analysis. S.F. performed experiments. N.T. contributed to data analysis and wrote the manuscript. P.T. and H.B. contributed to experimental design, performed experiments, contributed to data analysis and wrote the manuscript. All authors read and approved the final manuscript for publication.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.091082/-/DC1

References


Fig. S1. Characterisation of the mutated E14Dmras cell line and of ESCs overexpressing EGFP-MRAS protein. (A) Self-renewal capacity of E14WT and E14Dmras cell lines in clonal assay. 1, 3, 5, 10, 50, 100, 200 or 500 cells (as presented in A) were plated in triplicate in complete medium with LIF and stained using an alkaline phosphatase kit (pink coloration of cells) a week after plating. Efficiency of plating is 35% for the E14WT cell line and 7% for the E14Dmras cell line. Two independent experiments with samples in triplicate were performed. (B) Western blot analysis of control clone overexpressing EGFP (five independent clones) (lane 1) or clones expressing EGFP-MRAS (lane 2). Twenty clones that overexpress EGFP-MRAS, after 4-6 weeks in selective medium (DMEM with 10% FCS, 250 pM LIF and 300 µg/ml G418) were derived. The clones were amplified and grown with or without LIF for 3, 4 or 7 days. (C) Phase contrast (left) and fluorescence (right) images of a selection of individual EGFP and EGFP-MRAS clones grown in the presence or absence of LIF for 6 days. Scale bars: 100 µm.
Fig. S2. Sustained expression of OCT4 in EGFP-MRAS-overexpressing clones grown without LIF. Western blot analysis of OCT4 and ERK2 expression for a selection of clones grown with (+) or without (−) LIF for 3, 4 or 7 days (d3, d4 or d7). In clones that became heterogeneous for EGFP-MRAS expression after a few passages, fluorescent cells were sorted (on a FACSARia III cell sorter, Becton Dickinson) and grown with and without LIF as indicated. Of the 20 clones analysed, more than 60% showed a significant sustained expression of OCT4 when grown without LIF for 7 days.

Fig. S3. MRAS overexpression does not modulate the activation of MAPK and PI3K pathways. Western blot analysis of ESC clones expressing EGFP or EGFP-MRAS grown without LIF (−) for 24 hours (24h) and reinduced with LIF (+) for 30 minutes (induction LIF) in the continuous presence (+) or absence (−) of LIF for 4 days (4d). The specific antibodies used are indicated.
**Fig. S4.** *Mras* is re-expressed in ESC-derived mature neurons. Heat map analysis of Ras family members (same list as in Fig. 1) during the neuronal differentiation procedure. Embryoid bodies of 14TG2a-Sox1 ESCs have been treated with retinoic acid (10⁻⁶ M) as described (Abranches et al., 2009). This procedure leads to mature Tuj1-positive neurons. Microarray data are from published sources (Abranches et al., 2009; Schultz et al., 2009).
**Fig. S5. Inhibition of Mras mRNA translation by morpholinos.** Mras mRNA was translated *in vitro* in the presence of [35S]methionine and 40 ng (+) or 400 ng (++) morpholinos directed against Mras (Mras MO) or 400 ng of a standard control (Std) or mismatch (Mis) morpholino. Translation products were analysed by SDS-PAGE followed by autoradiography. Lane c contains a blank translation (no mRNA). MRAS is 24 kDa.

**Fig. S6. Mras stimulates neuronal differentiation of pluripotent animal cap cells.** Two-cell stage *Xenopus* embryos were injected with 500 pg Mras mRNA with (+) or without (−) 50 pg noggin mRNA and animal caps were dissected at stage 8 (Early) or stage 11 (Late) and analysed by RT-PCR when the control embryo (E) had reached stage 23 for N-tubulin (*N-tub*) and N-cam expression. Odc was used as loading control.
### Table S1. Primers for RT-qPCR and semi-quantitative PCR

#### RT-qPCR

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