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# Spatio-temporal expression pattern of the NatB complex, Nat5/Mdm20 in the developing mouse brain: Implications for co-operative *versus* non-co-operative actions of Mdm20 and Nat5

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ABSTRACT

The NatB complex, Nat5/Mdm20 acetyltransferase mediates *N*-acetylation to control cell cycle progression and actin dynamics in yeast. As yet, little is known about the expression pattern of Mdm20 and Nat5 in multi-cellular organisms. Here we show that Mdm20 is highly expressed in mouse embryonic brain. At E11.5, Mdm20 was widely expressed in both neural progenitors and early differentiating neurons, whereas Nat5 was expressed in Sox1/3+/Mdm20+ neural progenitors. By E14.5, both Mdm20 and Nat5 were downregulated in most ventricular zone neural progenitors, whereas both proteins were found in differentiating neurons and co-expression was maintained at E18.5 in derivatives of these cells, such as midbrain dopaminergic (DA) neurons and septal neurons. These data suggest that Nat5/Mdm20 complex-mediated acetylation may play a role in the proliferation and differentiation of neural progenitors. Intriguingly, our data also showed that Mdm20 is not always co-expressed with Nat5 in all differentiated neurons, for example deep cerebellar neurons. Moreover, detailed examination of the subcellular localization of Mdm20 and Nat5 in cultured Nat5+/Mdm20+ midbrain DA neurons revealed that Mdm20 is also not necessarily co-localized with Nat5 within neurons. Given that Nat5 is only a known member of Nat family protein that interacts with Mdm20, our data imply that Mdm20 may function either with an unidentified Nat protein partner(s) or possibly in a Nat-independent manner.



Post-translational modification of proteins is critically important in biology and expands both the structural and functional diversity of proteins, contributing to both evolutionary divergence and population diversity (Magalon et al., 2008; Arnesen et al., 2009). Two particular protein modifications, acetylation and phosphorylation, play key roles in a wide range of biological events, including tissue development (Kouzarides, 2000; Choudhary et al., 2009; Sadoul et al., 2010). The sculpting of tissue morphology over time is also dependent on the spatial and temporal control of gene transcription (Dessaud et al., 2007; Ohyama et al., 2008; Pearson et al., 2011), and indeed acetylation and phosphorylation often co-operate to modulate protein activities, which in turn modulate gene transcription (Sims and Reinberg, 2008; Lau and Cheung, 2011). For instance, a lysine acetyltransferase (KAT) – formerly called HAT (histone acetyltransferase) – mediates acetylation of the internal lysine residues of histones which, coupled with histone phosphorylation and methylation, controls the de-repression of polycomb-silenced genes, whose temporally-regulated functions are crucial to development (Lau and Cheung, 2011). Conversely, nuclear-located histone deacetylases (HDACs) mediate the repression of gene transcription by the polycomb complex (Sadoul et al., 2010; Garrick et al., 2008).

Recent studies extended the view that reversible acetyl modification of proteins at internal lysine residues is not limited to events in the nucleus but also occurs in the cytoplasm, controlling important biological processes such as translation, cellular apoptosis, motility, and protein quality control (Creppe et al., 2009; Ctalano et al., 2007; Kim et al., 2006; Sadoul et al., 2010). For instance, αTAT1 is responsible for the acetylation of α-tubulin, whereas HDAC6 functions as a α-tubulin deacetylase exclusively in the cytoplasm (Shida et al., 2010; Hubbert et al., 2002). As a consequence, these modifications regulate microtubule stability, and thus cell motility, independent of both histone metabolism and gene transcription (Hubbert et al., 2002). While to date several hundreds of cytosolic proteins have been found to undergo an acetyl modification, the biological significance of this remains to be elucidated (Kim et al., 2006; Choudhary et al., 2009).

N-terminal acetylation is another enzyme-catalyzed reaction whereby N-terminal residues accept the acetyl group from acetyl-CoA. While it takes a place on approximately 80–90% of

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cytosolic proteins in mammals, less attention has been paid to its function (Ametzazurra et al., 2008; Polevoda and Sherman, 2003; Polevoda et al., 2008, 2009). Emerging evidence suggests that an N-terminal acetylation of nascent polypeptides synthesized on polyribosomes plays a pivotal role in cellular homeostasis (Starheim et al., 2008; Arnesen et al., 2010). Based on *in vitro* studies, this modification has been proposed to influence protein function, stability, and subsequent modifications that include phosphorylation. It has been shown to control cell proliferation and protein quality to prevent the aggregation of abnormal proteins (Starheim et al., 2008; Arnesen et al., 2010). Biochemical studies have also shown that in the nervous system serotonin undergoes N-terminal acetyl modification and subsequent conversion to melatonin, thereby regulating the synthesis and metabolism of serotonin as a feedback system (Issac et al., 1990; Miguez et al., 1997; Sugiura et al., 2003). Nonetheless, due to the shortage of *in vivo* studies, it is still not well understood how N-terminal acetylation controls biological events in tissues.

*N*-acetyltransferase (Nat) complexes are composed of catalytic and auxiliary subunits, and are responsible for the enzymatic reaction to acetylate cytosolic proteins (Ametzazurra et al., 2008; Polevoda and Sherman, 2003; Polevoda et al., 2008, 2009). In yeast, five *N*-alpha-acetyltransferases (NATs), i.e. NatA-E, have been described as catalytic subunits, depending on the distinct amino acid sequences at N-termini they recognize. NatA, NatB, and NatC are three major *N*-acetyltransferases, whereas the substrates for NatD and E are poorly identified. As auxiliary subunits of three major Nat complexes (i.e. NatA, NatB, and NatC), Nat1p, Mdm20p, Mak31p have been identified, respectively in yeast.

In mammals, the NatA complex is composed of a catalytic subunit, ARD1 (arrested defective 1) and an auxiliary subunit NATH (*N*-acetyltransferase human). In the developing mouse brain, both ARD1 and NATH are highly expressed in proliferating progenitors and their expression is downregulated as they differentiate (Gendron et al., 2000; Sugiura et al., 2003). A recent study also showed that NatA complex ARD1-NAT1 is required for the dendritic arbolization of Purkinje cells in the postnatal cerebellum (Ohkawa et al., 2008). These studies clearly indicate that N-terminal acetyl-modification of proteins plays an important role in both the developing and mature brain.

Mdm20 (a regulator of mitochondrial distribution and morphology) is an auxiliary subunit of the NatB complex, the second major acetyltransferase and binds catalytic subunit Nat3 in yeast (Starheim et al., 2008; Ametzazurra et al., 2008; Polevoda and Sherman, 2003; Polevoda et al., 2009). The yeast NatB complex, Mdm20/Nat3 regulates tropomyosin-actin interactions (Singer and Shaw, 2003). In human cells, the NatB acetyltransferase complex is composed of Mdm20 and Nat5 (the human orthologue of yeast Nat3) and has been shown to be essential for cell cycle progression. (Starheim et al., 2008). Given that mouse Nat5 is the closest Nat family protein to human Nat5 and yeast Nat3, mMdm20 and mNat5 are likely to be the auxiliary and catalytic subunits of a mouse NatB acetyltransferase complex, respectively (Starheim et al., 2008; Ametzazurra et al., 2008; Polevoda and Sherman, 2003; Polevoda et al., 2008).

Although *in vitro* studies have suggested the importance of NatB complexes in fundamental cellular events, namely cell proliferation and cytoskeletal organization, none of the subunit proteins

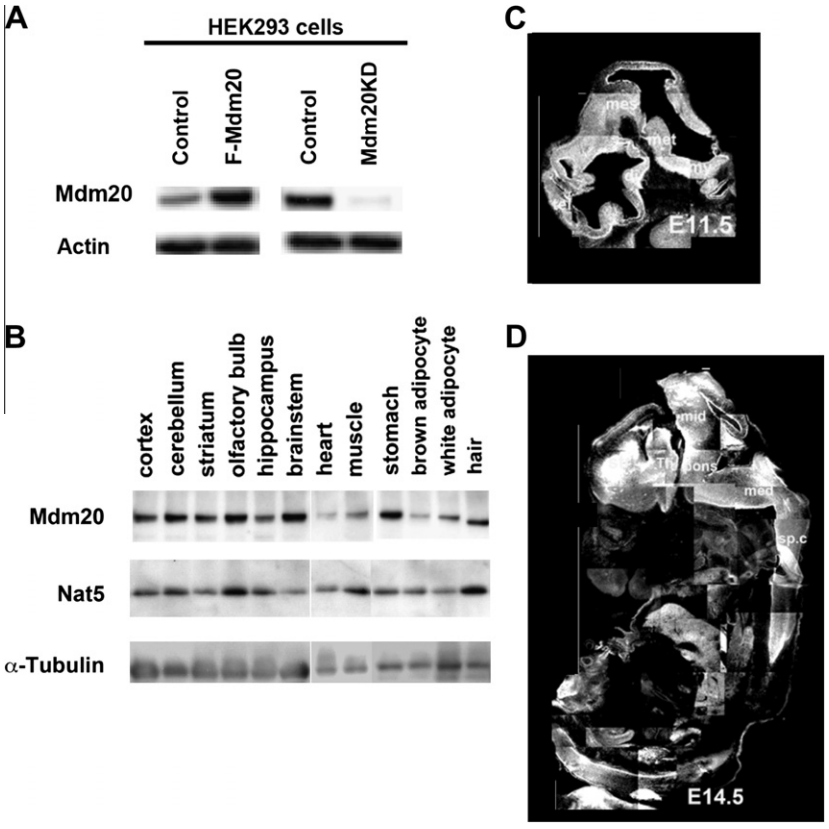


**Fig. 1.** Mdm20 is highly expressed in the embryonic and adult mouse brains. (A) Western blot of human embryonic kidney (HEK) 293 cells lysates using anti-Mdm20 rabbit polyclonal antibody. When a flag-tagged human Mdm20 was overexpressed (F-Mdm20), an increased expression of Mdm20 was detected. Conversely, an endogenous expression of human Mdm20 was decreased when human Mdm20 was knocked down by human Mdm20 siRNA (Mdm20KD). Western blot of β-actin was used as an internal control. (B) Western blot analysis of adult mouse tissues. Both Mdm20 and Nat5 are highly expressed in the brain (cerebral cortex, cerebellum, striatum, olfactory bulb, hippocampus, brainstem), stomach, and hair, compared to their expression level in heart and muscle. α-Tubulin expression was monitored as internal controls. (C and D) Immunofluorescent labeling of Mdm20 in mouse embryo at E11.5 (C) and E14.5 (D). Sagittal section of the mouse embryonic brain shows that Mdm20 is highly expressed in the brain and spinal cord. tel; telencephalon, mes; mesencephalon, dien; diencephalon, bs; brainstem, str; striatum, sp.c; spinal cord.

have been investigated for their function *in vivo*. In fact, the expression patterns of Mdm20 and Nat5 have not been examined systematically in multi-cellular organisms. Here we document for the first time the distribution pattern of Mdm20 protein in the developing mouse brain and compare it with that of Nat5. We further describe their subcellular localization and discuss their possible functions in the developing brain.

## 1. Results

### *1.1. Mdm20 is highly expressed both in the developing and adult mouse brain*

We first generated a rabbit polyclonal antibody against a carboxy-terminal sequence of the Mdm20 protein conserved between human and mouse. Using human embryonic kidney (HEK) 293 cell lysates, we performed Western blot analysis of Mdm20 and detected a specific single band, which corresponds to the predicted molecular weight 120 kD (Fig. 1 and data not shown). The anti-Mdm20 antibody also detected either the increase or decrease of Mdm20 expression when Flag-tagged full length of Mdm20 cDNA (F-Mdm20) was overexpressed or Mdm20 was knocked down by siRNA for Mdm20 (Mdm20KD), respectively (Fig. 1A). These data confirmed the specificity of the anti-Mdm20 antibody.

We next examined the tissue distribution of Mdm20 protein in adult mice. Western blot analysis demonstrated that both Mdm20 and Nat5 were highly expressed in the brain, stomach, and hair (Fig. 1B). Similar to its prominent expression in the adult brain, Mdm20 was found to be highly expressed in the embryonic brain as evidenced by immunofluorescent staining (Fig. 1C and D). Mdm20 expression was also evident in dorsal root ganglia, muscles, chondrocytes, skin, and heart (data not shown).

### *1.2. Mdm20 and Nat5 expression in neural progenitors and differentiating neurons of mouse embryonic brain*

As Mdm20 is a component of the NatB complex, we further investigated the expression pattern of Mdm20 and compared it with that of Nat5 in the developing mouse brain. At E11.5 Mdm20 was widely expressed, including in most proliferating cell nuclear antigen (PCNA)+ proliferating neural progenitors (Fig. 2A–F). By contrast, somewhat surprisingly, Nat5 expression was considerably more restricted, notably in only a subset of PCNA+ neural progenitors (Fig. 2A–F). Double labelling of Nat5 and Sox1/3 revealed that Nat5+ cells were Sox1/3+ neural progenitors (Fig. 3). Consistent with this, pulse labeling with BrdU revealed that Mdm20+ cells at the VZ were BrdU+ proliferating progenitors (Fig. 4A). Our data also showed that Mdm20+ cells in the mantle

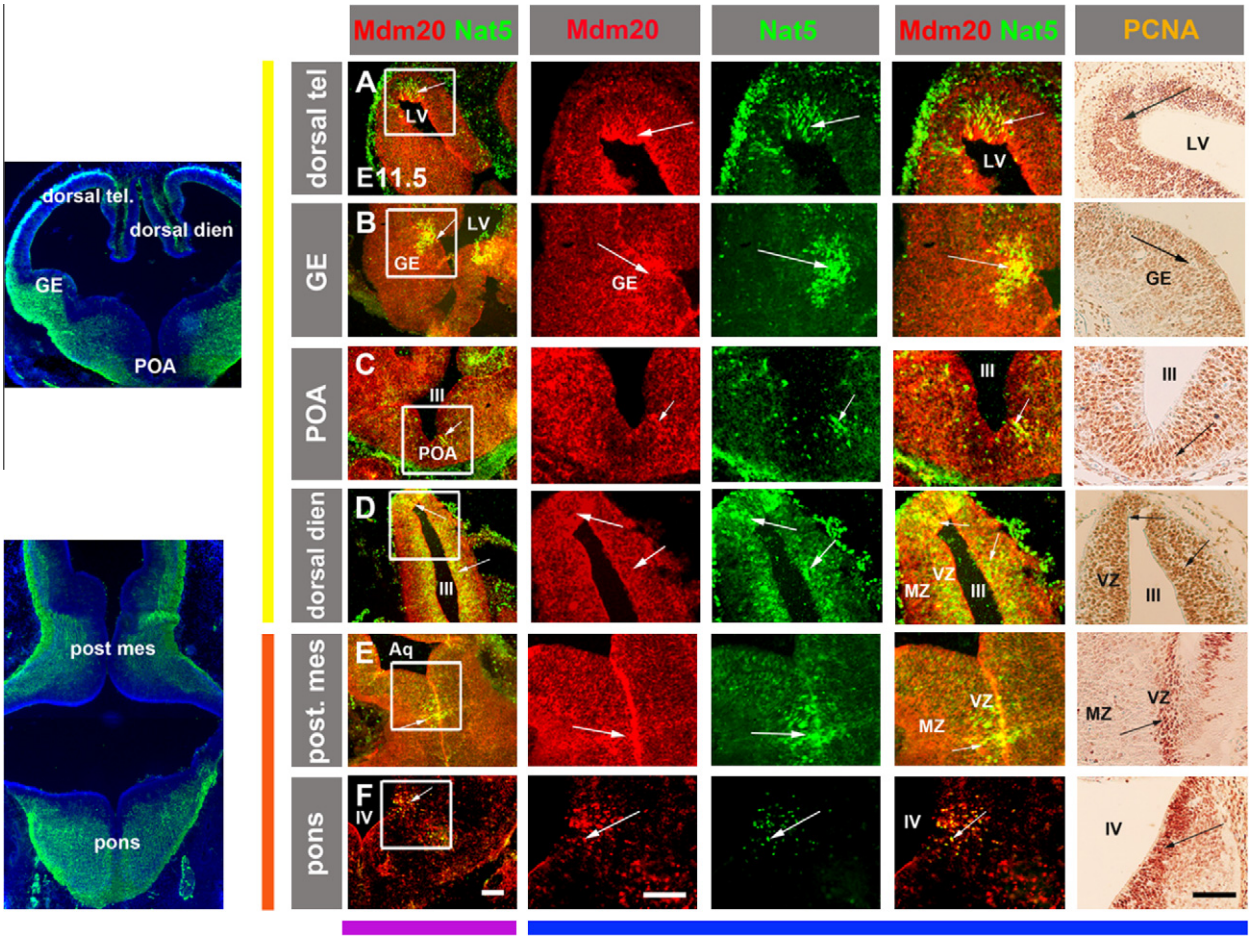


**Fig. 2.** Expression of Mdm20 and Nat5 in mouse embryonic brain at E11.5. (A–F) Transverse sections through the brain show a widely distributed expression of Mdm20 (red). In contrast, Nat5 expression (green) is restricted to subsets of proliferating cell nuclear antigen (PCNA)+ proliferating neural progenitors at the ventricular zones (VZ) of dorsal telencephalon (tel) (arrows in A), GE and POA in the ventral telencephalon (arrows in B and C), dorsal diencephalon (dien) (arrows in D), posterior mesencephalon (post mes) (arrows in E), and at the border between alar and basal plates of the pons (arrows in F). Nat5 expression was also found in the head mesenchyme. Left panels show TuJ1+ early neurons (green) at lower magnification of the brain tissues with DAPI counter stain (blue). Boxed regions were shown as at higher magnification. PCNA staining was performed at the equivalent levels but in different brain tissues from those for the analysis of Mdm20 and Nat5 expression. LV, lateral venricle; GE, ganglionic eminence; III, the third ventricle; VZ, ventricular zone; MZ, mantle zone; POA, preoprtic area; Aq, aqueduct; IV, the fourth ventricle. Scale bars: 30 μm.

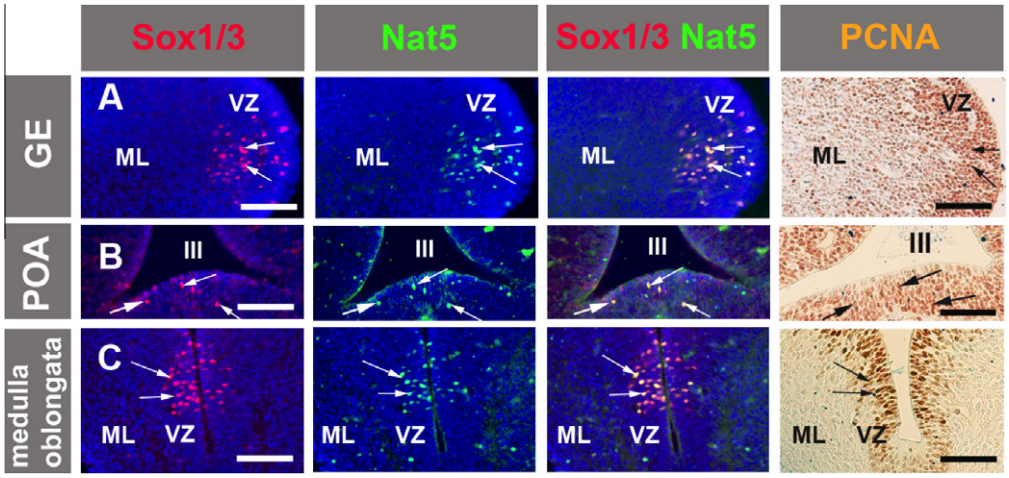


**Fig. 3.** Nat5 expression in Sox1/3+ neural progenitors at E12.5. (A–C) Transverse sections through the ganglionic eminence (GE), preoptic area (POA), and medulla oblongata at E12.5. Co-expression of Sox1/3 and Nat5 is found in PCNA+ proliferating progenitors at the VZ of the GE, POA, and medulla oblongata (arrows in A–C). GE, ganglionic eminence; POA, preoprtic area; VZ, ventricular zone; ML, mantle layer. III, the third ventricle. Scale bars: 30 μm

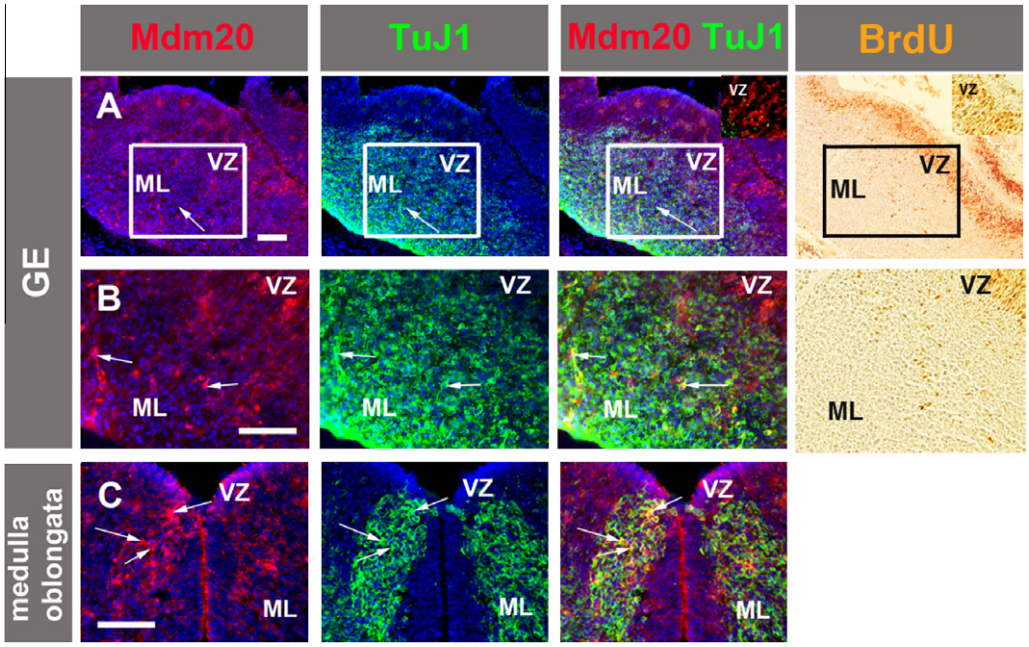


**Fig. 4.** Mdm20 is expressed in both proliferating neural progenitors at the VZ and TuJ1+ early differentiating neurons at E12.5. (A–C) Transverse sections through the GE and the medulla oblongata. Mdm20 is expressed both in the VZ and ML of both the GE and the medulla oblongata (A–C). Bromodeoxy-uridine (BrdU) pulse-labeled cells for 1hr are located mostly in the VZ (A and B). Mdm20+ cells co-express an early neuronal marker TuJ1 (arrows, A–C). GE, ganglionic eminence; POA, preoprtic area; VZ, ventricular zone; ML, mantle layer. Scale bars: 30 μm.

layer (ML) were TuJ1+, indicating that they are early differentiating neurons (Fig. 4A–C).

By E14.5, Mdm20/Nat5 co-expression was found in microtubule-associated protein 2 (MAP2)+ differentiating neurons of the following brain regions: olfactory bulb, septum, ganglionic eminence (GE) and its caudal part, CGE, which gives rise to amygdaloid neurons. Other regions that co-express Mdm20 and Nat5 include the preoptic area (POA), bed nucleus stria terminalis (BNST), zona incerta (Zi), epithalamus (Epith), dorsal raphe (DR), principal sensory nucleus of trigeminal nerves (Pr5s), vestibular nucleus, cochlear, external cuneate nucleus (EC), medullary raphe (Ra), spinal trigeminal nucleus (Sp5n) and so on (Figs. 5 and 6). It is noteworthy that cerebellar deep neurons express Mdm20 but not Nat5 (Fig. 6E), indicating the existence of Mdm20+/Nat5− cells in the brain.

At E18.5, co-expression of Mdm20/Nat5 was maintained in differentiated neurons such as those in the septum, pineal gland, piriform cortex, accumbens nucleus (NAc), Zi, amygdala, cingulate cortex, ventral tegmental area, interpeduncular nucleus, dorsal raphe, and medullary raphe (Ra) containing serotonergic (5-HT) neurons, spinal trigeminal nucleus (Pr5n), cochlear nucleus, pontine reticular formation (RF), external cuneate nucleus (EC), and inferior olivary nucleus (IO) (Figs. 7 and 9, and data not shown).

### *1.3. Mdm20 is not necessarily co-expressed with Nat5 in the developing mouse brain*

At early stages of neurogenesis (E11.5–12.5), the majority of neural progenitors at the VZ that express Mdm20 do not co-express Nat5 (Fig. 2). Similarly, while a vast majority of Nat5+

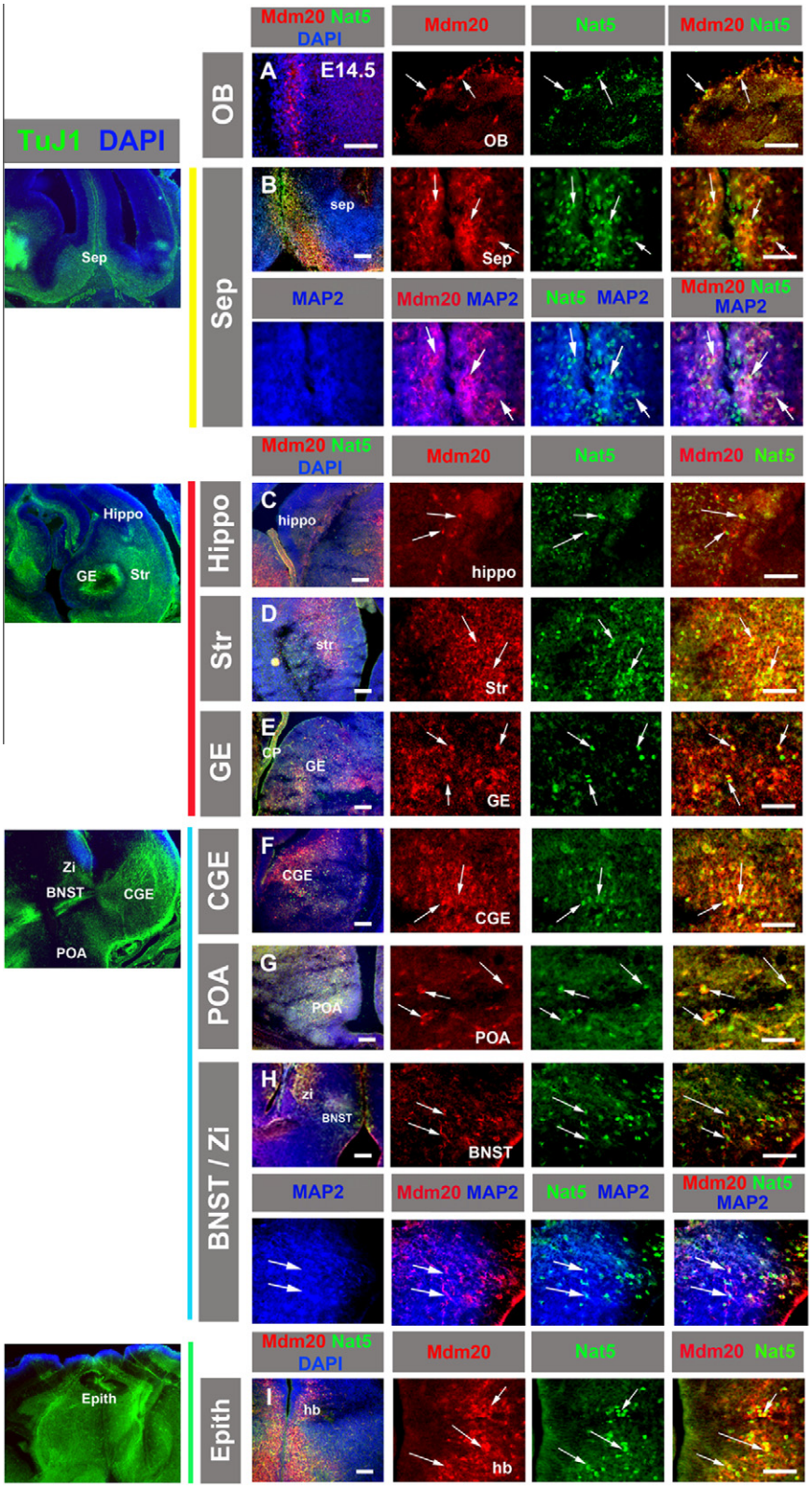


**Fig. 5.** Expression of Mdm20 and Nat5 in differentiating neurons of mouse embryonic forebrain at E14.5. (A–I) Transverse sections through the mouse forebrain at E14.5. Co-expression of Mdm20 and Nat5 is found in differentiating neurons of the following forebrain regions (arrows) in A–I: (A) olfactory bulb (OB); (B) septum (Sep); (C) hippocampus (Hippo); (D) striatum (Str); (E) ganglionic eminence (GE); (F) caudal ganglionic eminence (CGE); (G) preoptic area (POA); (H) bed nucleus stria terminalis (BNST)/zona incerta (Zi); epithalamus (Epith). Left panels show TuJ1+ early neurons (green) at lower magnification of the brain regions counterstained with DAPI (blue). Co-expression of Mdm20, Nat5, and neuronal marker MAP2 was found in the Sep (arrows in B) and BNST (arrows in H). Scale bars:30 μm on the left column of A–I; 15 μm on the three columns of A-I from the right, showing pictures at a higher magnification.

differentiating neurons co-express Mdm20 at E14.5 and onward, Mdm20+/Nat5− neurons were also observed in the brain regions such as facial nucleus, preoptic area (POA), thalamic paraventricular nucleus (Th PVN), lateral reticular nucleus (LRN), and cerebellar

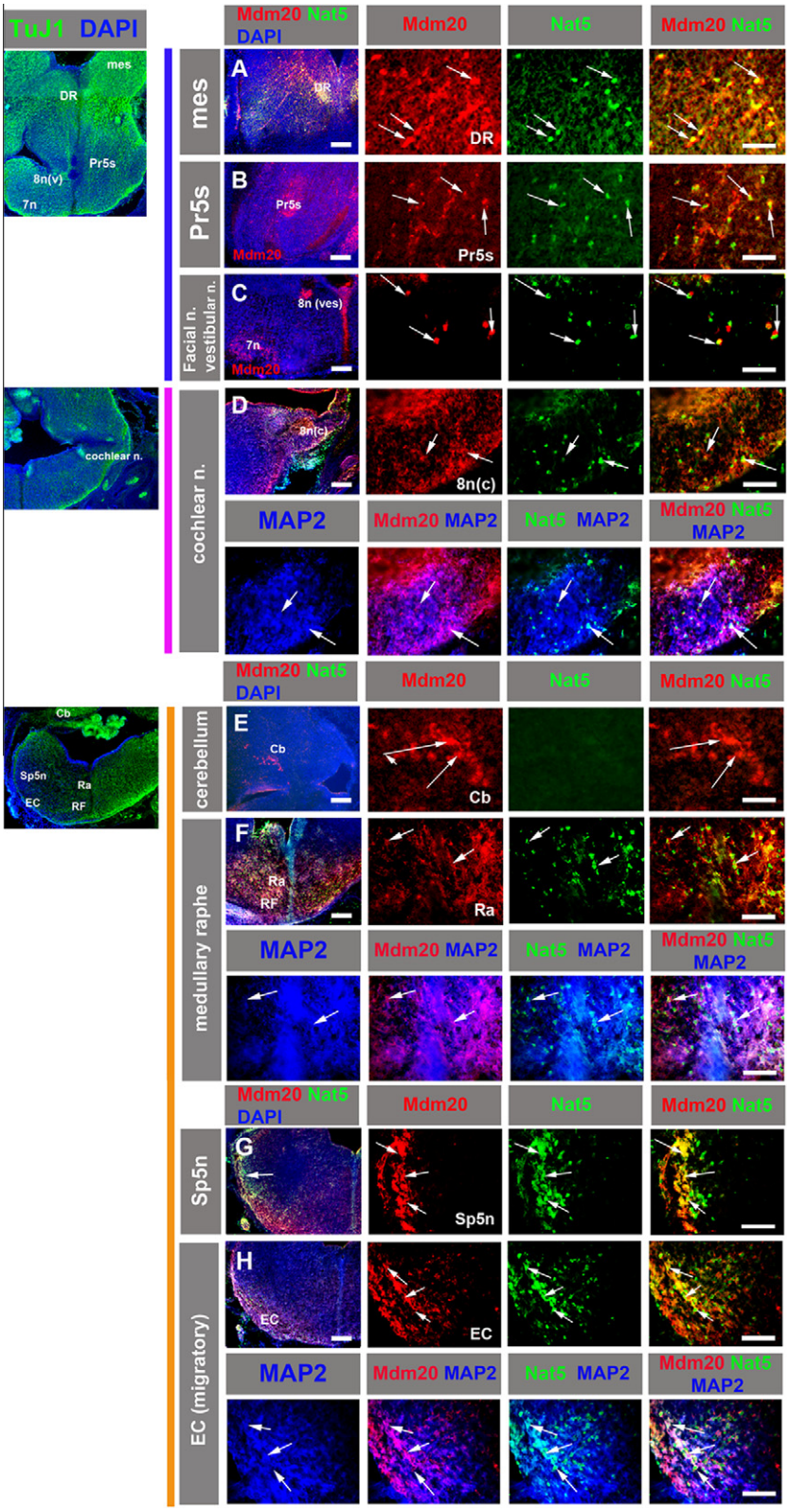


**Fig. 6.** Mdm20 and Nat5 expression in differentiating neurons of mouse brainstem at E14.5. (A–H) Transverse sections through the mouse brainstem at E14.5. (A–D) Co-expression of Mdm20 (red) and Nat5 (green) in the upper brainstem (arrows): (A) dorsal raphe in the mesencephalon (DR); (B) principal sensory trigeminal nucleus (Pr5s); (C) vestibular nucleus, Note that facial neurons does not express Nat5; (D) cochlear neurons. (E–H) Expression of Mdm20 (red) and Nat5 (green) in the lower brainstem and cerebellum (arrows): (E) cerebellar deep neurons (Cb); (F) medullary raphe neurons (Ra); reticular formation (RF); (G) spinal trigeminal neurons (Sp5n) (arrows); (H) migratory external cuneate neurons (EC) (arrows). Note that cerebellar deep neurons express Mdm20 but not Nat5. Left panels show TuJ1+ early neurons (green) at lower magnification of the brain regions stained wih DAPI (blue). Co-expression of Mdm20, Nat5, and neuronal marker MAP2 was found in the cochlear nucleus (arrows in D), RF (arrows in F), and EC (arrows in H). Scale bars: 30 μm on the left column of A–H; 15 μm on the three columns of A–H from the right, showing pictures at a higher magnification.

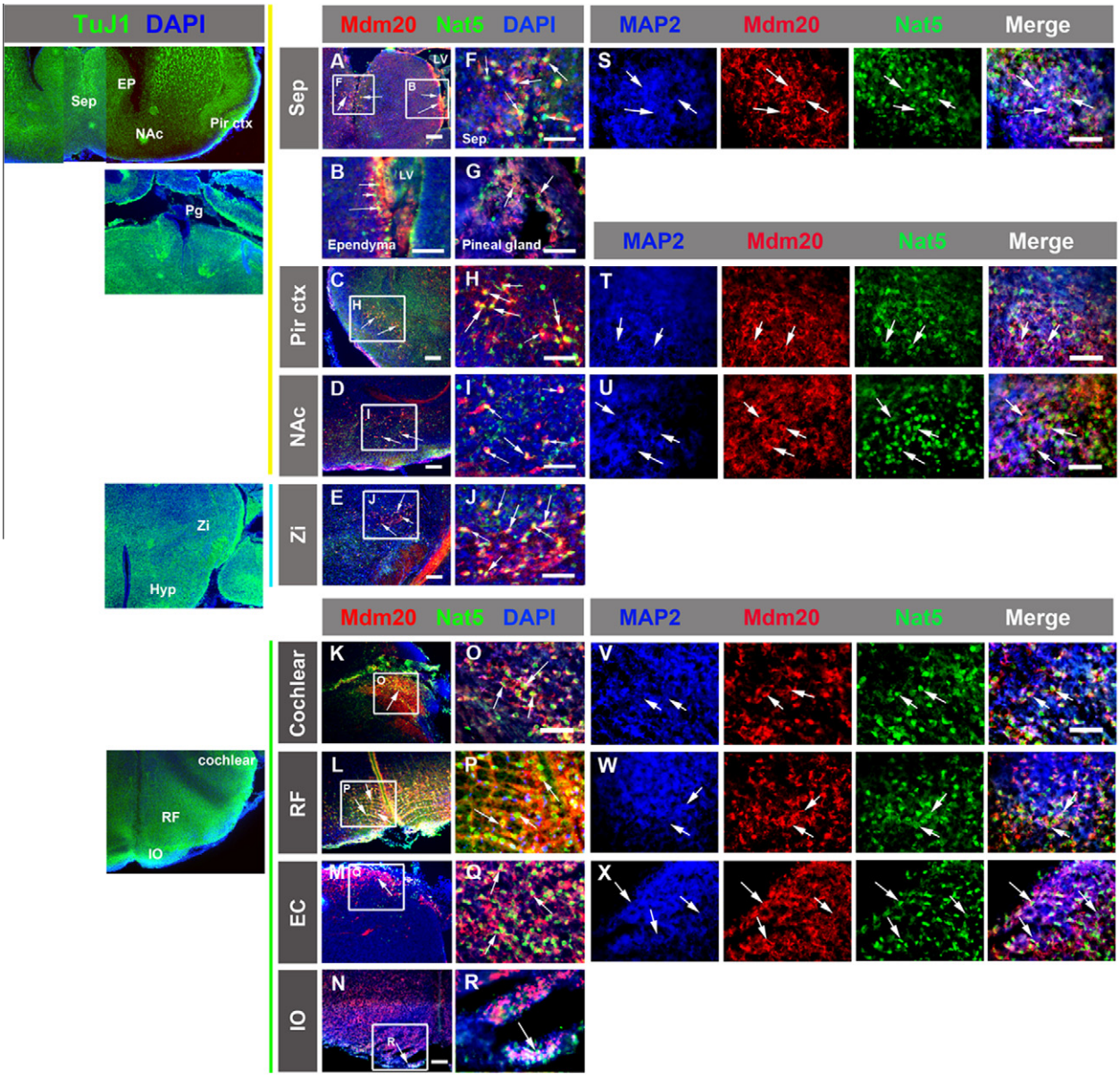


**Fig. 7.** Mdm20 and Nat5 are co-expressed in differentiated neurons at E18.5. (A–R) Transverse sections through the brain. Co-expression of Mdm20 and Nat5 is found in the septum (arrows in A and F), ependymal cells facing at the lateral ventricle (LV) (arrows in B), piriform cortex (Pir ctx) (arrows in C and H), accumbens nucleus (NAc) (arrows in D and I), zona incerta (Zi) (arrows in E and J), pineal gland (Pg) (arrows in G), cochlear nucleus (arrows in K and O), potine reticular formation (RF) (arrows in L and P), EC (arrows in M and Q), and the ventral portion of inferior olivary nucleus (IO) (arrows in N and R). Left panels show TuJ1+ neurons (green) at a lower magnification of the brain regions stained wih DAPI (blue). hyp; hypothalamus. Co-expression of Mdm20, Nat5, and neuronal marker MAP2 was found in the Sep (arrows in S), Pir ctx (arrows in T), NAc (arrows in U), cochlear nucleus (arrows in V), RF (arrows in W), and EC (arrows in X). Scale bars: 30 μm in A, C, D, E, K–N; 15 μm in B, F-J, O–X.

deep neurons (Figs. 6C and E and 8A–D). Moreover, although vestibular neurons (Ves) co-express Mdm20 and Nat5 at E14, the downregulation of Nat5 occurs in the Ves by E18.5 (Figs. 6C and 8B). These data indicate that the expression of Mdm20 and Nat5 is not only spatially regulated but also changes over time.

Although Mdm20 and Nat5 were found to co-express in the midbrain DA neurons *in vivo* (Fig. 9A–C), we also noted that their subcellular localization appeared to be distinct (Fig. 9A and B). Mdm20 was mainly localized in the cytoplasm, whereas Nat5 was found in the nucleus. This data is consistent with the previous observation using cultured tumor cells (Starheim et al., 2008), although they showed some co-localization of Mdm20 and Nat5 in the cytoplasm. To clarify the subcellular localization of Mdm20 and Nat5 in neurons, we monitored their expression in the midbrain DA neurons cultured *in vitro*. The midbrain was isolated from E18.5 rat embryos and cultured for 2–4 weeks. Expression of Mdm20 and Nat5 was assessed by an immunofluorescent labeling experiment. Consistent with *in vivo* data (Fig. 9A–C), Mdm20 was mainly localized in the cytoplasm especially in the perinuclear region of tyrosine hydroxylase (TH)+ DA neurons, whereas Nat5 was mainly in the nucleus (Fig. 9D–G).

## 2. Discussion

In the present study, we have documented the spatial and temporal expression pattern of Mdm20 in comparison with that of Nat5 in the developing mouse brain. Mdm20 is highly expressed in the developing mouse brain. Early on, it is widely distributed in neural progenitors (Figs. 1–4). As development proceeds, Mdm20 expression is downregulated in proliferating progenitors and found in differentiating neurons (Figs. 5–7). The vast majority

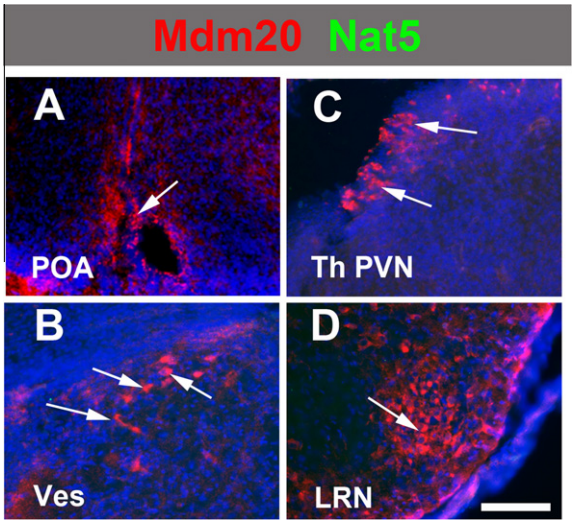


**Fig. 8.** Mdm20 and Nat5 are not necessarily co-expressed in neurons at E18.5. (A–D) Transverse sections through the brain at E18.5. Mdm20 (red) but not Nat5 (green) is found in (A) the preoptic area (POA) (A), vestibular nucleus (B), thalamic paraventricular nucleus (Th PVN) (C), and lateral reticular nucleus (D). Scale bars: 30 μm.

of the Mdm20+ neurons co-express Nat5, suggesting that the Mdm20/Nat5 acetyltransferase complex plays a role in the differentiation of neurons. Careful analysis of the expression pattern of Mdm20 and Nat5 also reveals the existence of Mdm20+/Nat5− neural progenitors and differentiating neurons (Figs. 2 and 8), and their subcellular localization does not entirely overlap in differentiated neurons as seen in midbrain DA neurons (Fig. 9).

Previous studies of yeast and human cells *in vitro* showed that Mdm20 controls cell proliferation and actin dynamics (Singer and Shaw, 2003; Starheim et al., 2008). Consistent with this notion, Mdm20 is widely expressed in neural progenitors (at E11.5–12.5) (Figs. 1 and 2). Given that cell polarity of neural progenitors is crucial to control the number of neural progenitors and their differentiation (Huttner and Kosodo, 2005), further studies are needed to explore the function of Mdm20 in proliferating progenitors and their differentiation in the brain.

Our data has also revealed that both Mdm20 and Nat5 expression changes in space and time. Intriguingly, by E14.5, both Mdm20 and Nat5 expression is downregulated in most of the proliferating progenitors at the VZ and becomes restricted to postmitotic differentiating neurons (Figs. 5 and 6). Our observation of Nat5/Mdm20 co-expression in Sox1/3+ proliferating neural progenitors supports a widely accepted role for Mdm20/Nat5 in cell proliferation (Starheim et al., 2008). Moreover, our data imply that Mdm20/Nat5 has an unidentified additional role in differentiating neurons as we discuss below.

Biochemical studies previously showed that serotonin (5-HT) undergoes acetyl modification and conversion to melatonin in the pineal gland. However, it has been shown that Nat1/NATH1 does not mediate the *N*-acetylation of serotonin (Heim et al., 1991). In the present study, we found that Mdm20 and Nat5 are co-expressed in the pineal gland (Fig. 7G). It raises a possibility that

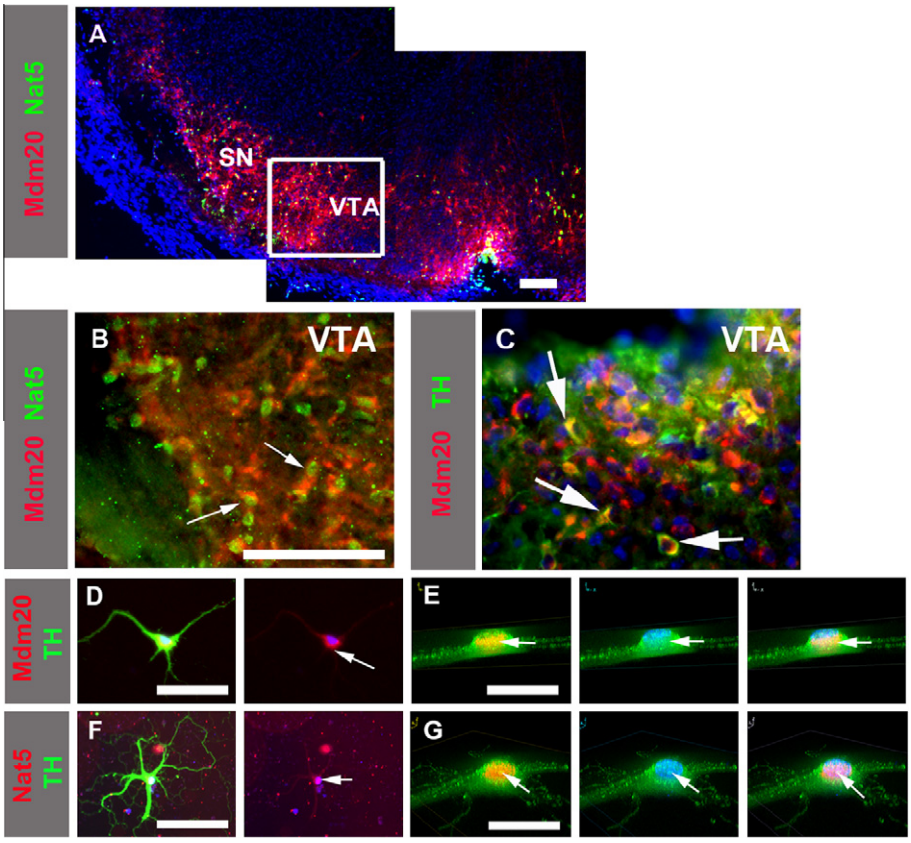


**Fig. 9.** Subcellular localization of Mdm20 and Nat5 protein in midbrain DA neurons. (A–C) Mdm20 (red) and Nat5 (green) are co-expressed in tyrosine hydroxylase (TH)+ dopaminergic (DA) neurons at the substantia nigra (SN) and the ventral tegmental area (VTA) in mouse midbrain (arrows). (D–I) Subcellular localization of Mdm20 (red) and Nat5 (red) in the rat midbrain DA neurons (green) cultured *in vitro*. To clarify the subcellular localization of both Mdm20 and Nat5, cell nuclei were visualized by DAPI staining (blue in E and G). Mdm20 (red) is mainly localized in the cytoplasm especially in the perinuclear region of the TH + DA neurons (arrows in D and E; note that Mdm20 is localized at the vicinity of DAPI+ cell nucleus), whereas Nat5 (red) is in the nucleus (arrows in F and G; note that Nat5 expression is overlapped with DAPI+ cell nucleus). Scale bars: 30μm in A–D, F; 5 μm in E and G.

the Nat5/Mdm20 complex contributes to the conversion of serotonin to melatonin in the gland, which needs to be tested in the future experiments.

More intriguingly, another possible role for Mdm20 and Nat5 is to control cellular survival and ageing (Liu et al., 2010; Arnesen et al., 2010), and we assume such a role should be very important in postmitotic cells, e.g. neurons. An assembly of actin cables is crucial for controlling cell polarity, and a tropomyosin-dependent polarized retrograde transport of protein aggregates along the actin cables is an important process for proteostasis (Liu et al., 2010). This process is mediated by a protein complex called a polarisome, which could be associated with Mdm20. As these processes are crucial to prevent the senescence of budding yeast (Liu et al., 2010), we assume that Mdm20 may play a similar role in protein clearance in mature and/or aging neurons, and thus their survival. To support the notion, a recent study showed that a chaperone-like protein HYPK physically interacts with NatA, preventing Huntingtin aggregation (Arnesen et al., 2010). It is conceivable that, after becoming post-mitotic, neurons possess a cellular machinery by which they tightly regulate a protein quality for their survival throughout life. As the expression of Mdm20/Nat5 continues into adulthood, Mdm20 may play a role in protein clearance or metabolism in the long life of post-mitotic neurons.

In addition to the co-expression of Mdm20 and Nat5, our data clearly indicate the existence of Mdm20+/Nat5− neurons. Our data show that differential expression of Mdm20 and Nat5 is achieved in three different ways: (1) spatially distinct expression of Nat5 and Mdm20; (2) temporal downregulation of Nat5 in Mdm20+ cells; (3) different subcellular localization of Mdm20 and Nat5. A previous study suggested that Mdm20 and Nat5 have functions other than those as a Mdm20/Nat5 complex in human cells (Starheim et al., 2008). Given that mNat5 is currently only one possible example of the Nat protein family that interacts with Mdm20 (Singer and Shaw, 2003; Polevoda and Sherman, 2003), our data imply that Mdm20 may function with some unidentified Nat proteins and/or in a Nat-independent manner.

## 3. Experimental procedures

### 3.1. Animals

All experiments were carried out according to the guidelines established by the Animal Welfare Committee of Nagasaki University. Pregnant wild-type C57BL/6J mice and rats were sacrificed by cervical dislocation under deep anesthesia with diethyl ether. Mouse and rat embryos were harvested at E11.5–18.5 and E18.5, respectively. They were processed for immunohistochemistry or culture experiments as described in the following Sections 3.3 and 3.4.

### 3.2. Antibody generation and Western blot analysis

A rabbit polyclonal antibody was generated against C-terminal peptides, LEDTSLSPEERKFSKTVQGKVQSSYLHSLLEMGELLKKRLETTKKLKI of human Mdm20 protein, which is conserved between human and mouse, fused with GST protein. The antiserum was purified by GST column and protein A sepharose. Western blot analysis of adult mouse tissues (20 μg of protein per lane) was carried out as described previously (Mori-Konya et al., 2009). Flow through fraction of antiserum with GST column did not detect the 120 kD band, confirming the specificity of the anti-Mdm20 antibody generated. Western blots of either α-tubulin or β-actin were used as internal controls. Mdm20 was knocked down by siRNA (Mdm20KD), whose target sequence was the 329–248 nucleotide sequence of human Mdm20 gene.

### 3.3. Immunohistochemistry

Mouse embryonic brains (C57BL/6J; $n = 5–8$ for each stages examined) were fixed in 4% paraformaldehyde (PFA) for 2–6 h depends on their age. After washing with PBS, tissues were immersed in 30% sucrose/0.1 M phosphate buffer (PB). Cryosections were incubated with antibodies as described previously (Ohyama et al., 2004, 2005). Pulse labeling with BrdU for 1 h was performed as described previously (Ohyama et al., 2004). Following antibodies were used: rabbit anti-Mdm20 polyclonal antibody (1:250); goat polyclonal anitibody against Nat5 (Santa Cruz, CA, USA, 1:25–100); TuJ1 mouse IgG (Covance, Japan, 1:1000); mouse anti-PCNA (Novocastra, U.K., 1:100); rabbit anti-Sox1/3 (1:1000, gift of H. Kondo); mouse anti-BrdU (Becton Dickinson, NJ, USA, 1:50–100); mouse anti-TH monoclonal antibody (Sigma, 1:2000); mouse anti-MAP2 (Sigma-Aldrich, MO, USA, 1:500–1000); Alexa 567 donkey anti-rabbit IgG (Molecular probes, USA, 1:500); Alexa 488 donkey anti-goat IgG (Molecular probes, USA, 1:500); Alexa 488 donkey anti-mouse IgG (Molecular probes, USA, 1:500); Alexa 305 anti-mouse IgG (Molecular probes, USA, 1:500); HRP-conjugated anti mouse IgG. Images were captured using fluorescent and confocal microscope (Carl Zeiss Axioskop2 plus; Carl Zeiss Axiovert200M LSM510) with an Axiovision software and BIOREVO BZ-8100 imaging system (Keyence, Osaka, Japan). Primary antibody incubation was omitted for negative control experiments. The specificity of Nat5 immunoreactivity was also confirmed by neurtralization with blocking peptide (Santa Cruz, CA, USA) following a manufacturer's instruction. The specificity of anti-Mdm20 sera was also confirmed by neutralization with purified Mdm20 antigen (data not shown).

### 3.4. In vitro culture of midbrain DA neurons

Embryonic midbrain tissues were isolated from pregnant rats (Sprague Dawley) at E18.5, and were cultured using a standard protocol as described previously for hippocampal neurons (Morii et al., 2006).

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