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The long non-coding RNA Paupar promotes KAP1-dependent chromatin changes and regulates olfactory bulb neurogenesis

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Abstract

Many long non-coding RNAs (lncRNAs) are expressed during central nervous system (CNS) development, yet their in vivo roles and mechanisms of action remain poorly understood. Paupar, a CNS-expressed lncRNA, controls neuroblastoma cell growth by binding and modulating the activity of transcriptional regulatory elements in a genome-wide manner. We show here that the Paupar lncRNA directly binds KAP1, an essential epigenetic regulatory protein, and thereby regulates the expression of shared target genes important for proliferation and neuronal differentiation. Paupar promotes KAP1 chromatin occupancy and H3K9me3 deposition at a subset of distal targets, through the formation of a ribonucleoprotein complex containing Paupar, KAP1 and the PAX6 transcription factor. Paupar-KAP1 genome-wide co-occupancy reveals a fourfold enrichment of overlap between Paupar and KAP1 bound sequences, the majority of which also appear to associate with PAX6. Furthermore, both Paupar and KAP1 loss-of-function in vivo disrupt olfactory bulb neurogenesis. These observations provide important conceptual insights into the trans-acting modes of lncRNA-mediated epigenetic regulation and the mechanisms of KAP1 genomic recruitment, and identify Paupar and KAP1 as regulators of neurogenesis in vivo.

Keywords chromatin; gene regulation; KAP1; lncRNA; neurogenesis

Subject Categories Chromatin, Epigenetics, Genomics & Functional Genomics; Neuroscience; RNA Biology

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Introduction

A subset of nuclear long non-coding RNAs (lncRNAs) have been shown to act as transcription and chromatin regulators using various mechanisms of action. These include local functions close to the sites of lncRNA synthesis (Engreitz et al, 2016) as well as distal modes of action across multiple chromosomes (Chalei et al, 2014; Vance et al, 2014). Moreover, lncRNA regulatory effects may be mediated by the act of lncRNA transcription as well as RNA sequence-dependent interactions with transcription factors and chromatin-modifying proteins (Vance & Ponting, 2014; Rutenberg-Schoenberg et al, 2016). Some lncRNAs have been proposed to act as molecular scaffolds to facilitate the formation of multicomponent ribonucleoprotein regulatory complexes (Tsai et al, 2010; Zhao et al, 2010; Ilik et al, 2013; Maenner et al, 2013; Yang et al, 2014b), whilst others may act to guide chromatin-modifying complexes to specific binding sites genome-wide (Vance & Ponting, 2014). Studies of cis-acting lncRNAs such as Haunt and Hottip have shown that lncRNA transcript accumulation at their sites of expression can effectively recruit regulatory complexes (Yin et al, 2015; Pradeepa et al, 2017). LncRNAs, however, have also been reported to directly bind and regulate genes across multiple chromosomes away from their sites of synthesis (Chu et al, 2011; Chalei et al, 2014; Vance et al, 2014; West et al, 2014; Carlson et al, 2015). By way of contrast, the mechanisms by which such trans-acting lncRNAs mediate transcription and chromatin regulation at distal bound target genes are less clear.

LncRNAs show a high propensity to be expressed in various brain regions and cell types relative to other tissues (Mercer et al, 2008, 2010; Ponjavic et al, 2009). The adult neurogenic stem cell-containing mouse subventricular zone (SVZ) generates neurons throughout life, contributes to brain repair and can be stimulated to limit damage, but is also a source of tumours (Bardella et al, 2016; Chang et al, 2016). During SVZ lineage progression, neural stem cells give rise to transit amplifying progenitors which in turn generate neuroblasts that migrate in the rostral migratory stream (RMS) to the olfactory bulbs (OB; Doetsch et al, 1999). The neuroblasts primarily become granule neurons that differentiate by extending long branched dendritic processes towards the glomerular layer (Petreranu & Alvarez-Buylla, 2002). There they integrate into and...
modulate circuitry connecting peripheral olfactory receptor neurons with the output neurons of the OB (Gheusi et al., 2000; Lledo & Saghatelian, 2005). It has been estimated that 8,992 lncRNAs are expressed in the SVZ neurogenic system, many of which are differentially expressed during SVZ/OB neurogenesis, suggesting that at least some of these transcripts may play regulatory roles (Ramos et al., 2013). However, only a minority of SVZ expressed lncRNAs have been analysed functionally and the full scope of their molecular mechanisms of action remain poorly understood.

Kap1 encodes an essential chromatin regulatory protein that plays a critical role in embryonic development and in adult tissues. Kap1−/− mice die prior to gastrulation while hypomorphic Kap1 mouse mutants display multiple abnormal embryonic phenotypes, including defects in the development of the nervous system (Cammas et al., 2008; Herzog et al., 2011; Shibata et al., 2011). Kap1 interacts with chromatin binding proteins such as HP1 and the SETDB1 histone-lysine N-methyltransferase to control heterochromatin formation and to silence gene expression at euchromatic loci (Iyengar & Farnham, 2011). Despite this fundamental role in epigenetic regulation, the mechanisms of Kap1 genomic targeting are not fully understood. Kap1 does not contain a DNA binding domain but was originally identified through its interaction with members of the KRAB zinc finger (KRAB-ZNF) transcription factor family. Subsequent studies, however, revealed that KRAB-ZNF interactions cannot account for all Kap1 genomic recruitment events. Kap1 preferentially localises to the 3′ end of zinc finger genes as well as to many promoters and intergenic regions in human neuronal precursor cells. A mutant Kap1 protein, however, that is unable to interact with KRAB-ZNFs still binds to promoters, suggesting functionally distinct subdomains (Iyengar et al., 2011). This work points to the presence of alternative, KRAB-ZNF-independent, mechanisms that operate to target Kap1 to a distinct set of genomic binding sites. We reasoned that this may involve specific RNA–protein interactions between Kap1 and chromatin-bound lncRNAs.

The CNS-expressed intergenic lncRNA Paupar represents an ideal candidate chromatin-enriched lncRNA with which to further define trans-acting mechanisms of lncRNA-mediated gene and chromatin regulation. Paupar is transcribed upstream from the Pax6 transcription factor gene and acts to control proliferation and differentiation of N2A neuroblastoma cells in vitro (Vance et al., 2014). Paupar regulates Pax6 expression locally, physically associates with Pax6 protein and interacts with distal transcriptional regulatory elements to control gene expression on multiple chromosomes in N2A cells in a dose-dependent manner. Here, we show that Paupar directly interacts with Kap1 in N2A cells and that together they control the expression of a shared set of target genes enriched for regulators of neural proliferation and differentiation. Our findings indicate that Paupar, Kap1 and Pax6 physically associate on chromatin within the regulatory region of shared target genes and that Paupar knockdown reduces both Kap1 chromatin association and histone H3 lysine 9 trimethylation (H3K9me3) at Pax6 co-bound locations. Genome-wide occupancy maps further identified a fourfold enrichment in the overlap between Paupar and Kap1 binding sites on chromatin, the majority of which (73%) are also estimated to be bound by Pax6. Our results also show that both Paupar and Kap1 loss-of-function in vivo disrupt SVZ/OB neurogenesis. We propose that Paupar and Kap1 are novel regulators of neurogenesis in vivo and that Paupar operates as a transcriptional cofactor to promote Kap1-dependent chromatin changes at a subset of bound regulatory elements in trans via association with non-KRAB-ZNF transcription factors such as Pax6.

**Results**

Paupar directly binds the Kap1 chromatin regulatory protein in mouse neural cells in culture

The lncRNA Paupar binds transcriptional regulatory elements across multiple chromosomes to control the expression of distal target genes in N2A neuroblastoma cells (Vance et al., 2014). Association with transcription factors such as Pax6 assists in targeting Paupar to chromatin sites across the genome. As Paupar depletion does not alter Pax6 chromatin occupancy (Vance et al., 2014), we hypothesised that Paupar may recruit transcriptional cofactors to Pax6 and other neural transcription factors to regulate gene expression. To test this, we sought to identify transcription and chromatin regulatory proteins that bind both Paupar and Pax6 in N2A cells in culture. In vitro-transcribed biotinylated Paupar was therefore immobilised on streptavidin beads and incubated with N2A cell nuclear extract in a pulldown assay. Bound proteins were washed, eluted and identified using mass spectrometry (Fig 1A). This identified a set of 78 new candidate Paupar-associated proteins that do not bind a control RNA of similar size, including 28 proteins with annotated functions in the control of gene expression that might function as transcriptional cofactors (Fig 1B and Dataset EV1).

We next performed native RNA-IP experiments in N2A cells to validate potential associations between the endogenous Paupar transcript and five gene expression regulators. These candidates were as follows: Rcor3, a member of the CoREST family of proteins that interact with the REST transcription factor; Kap1, a key epigenetic regulator of gene expression and chromatin structure; PPAN, a previously identified regulator of Pax6 expression in the developing eye; CHE-1, a polymerase II interacting protein that functions to promote cellular proliferation and block apoptosis; and ERH, a transcriptional cofactor that is highly expressed in the eye, brain and spinal cord.

The results revealed that the Paupar transcript, but not a non-specific control RNA, was > twofold enriched using antibodies against Rcor3, Kap1, ERH, PPAN or CHE1 compared to an IgG isotype control in a native RNA-IP experiment (Fig 1C). In addition, Paupar did not associate above background with SUZ12, EED and EZH2 Polycomb proteins used as negative controls. This served to further confirm the specificity of the Paupar lncRNA–protein interactions because Polycomb proteins associate with a large number of RNAs (Davidovich et al., 2015) and yet were not identified as Paupar interacting proteins in our pulldown assay. The endogenous Paupar transcript therefore associates with proteins involved in transcription and chromatin regulation in proliferating N2A cells.

To characterise Paupar lncRNA–protein interactions further, we used UV-RNA-IP to test whether Paupar interacts directly with any of these five cofactors. These data showed that Paupar, but not an U1snRNA control, is highly enriched using antibodies against Kap1 or Rcor3 compared to an IgG control (Fig 1D). A lower level of Paupar enrichment is found with CHE1, whereas ERH or PPAN does not appear to interact directly with Paupar (Fig EV1). Furthermore,
In vitro transcribed biotinylated Paupar
Streptavidin Beads
Incorporate With Nuclear Extract
Washes
Elution
MASS SPECTROMETRY

78 SPECIFIC PAUPAR ASSOCIATED PROTEINS

| GOID       | Term                                | Number In Set | Number In Reference | Corrected P-value |
|------------|-------------------------------------|---------------|---------------------|-------------------|
| GO:0006396 | RNA processing                       | 19            | 513                 | 3.35 x 10^-12     |
| GO:0008380 | RNA splicing                         | 13            | 237                 | 6.72 x 10^-10     |
| GO:0022613 | ribonucleoprotein complex biogenesis | 10            | 195                 | 5.87 x 10^-7      |
| GO:0010467 | gene expression                      | 28            | 3596                | 5.44 x 10^-6      |

Fold Enrichment NativeIP/igG

Fold Enrichment UV-RIP/igG

Paupar
Plus UV
Minus UV

IP: FLAG
WB: KAP1
WB: KAP1

INPUT
FLAG-PAX6
KAP1
FLAG-PAX6 + KAP1

INPUT
FLAG-PAX6
RCOR3
FLAG-PAX6 + RCOR3

WB: RCOR3
WB: PAX6

WB: PAX6

Figure 1.
the association of Paupar with either KAP1 or RCOR3 was reduced in the absence of UV treatment (Fig 1E). These results indicate that the endogenous Paupar transcript directly and specifically associates with RCOR3 and KAP1 transcriptional cofactors in neural precursor-like cells in culture.

As a first step to determine whether KAP1 or RCOR3 can act as PAX6-associated transcriptional cofactors, we performed immunoprecipitation experiments in N2A cells using transfected FLAG-tagged PAX6 and KAP1 or RCOR3 proteins. Immunoprecipitation of FLAG-PAX6 using anti-FLAG beads co-immuno-precipitated transfected KAP1 protein, but not RCOR3 (Fig 1F), suggesting that PAX6 and KAP1 are present within the same multicomponent regulatory complex. Consistent with this, a previous study showed that KAP1 interacts with PAX3 through the amino terminal paired domain, which is structurally similar in PAX6, to mediate PAX3-dependent transcriptional repression (Hsieh et al., 2006). Together, these results indicate that KAP1 may regulate Paupar and PAX6-mediated gene expression programmes.

**Paupar and KAP1 control expression of a shared set of target genes that are enriched for regulators of neuronal function and cell cycle in N2A cells**

KAP1 regulates the expression of genes involved in the self-renewal and differentiation of multiple cell types, including neuronal cells (Iyengar & Farnham, 2011), and thus is an excellent candidate interactor for mediating the transcriptional regulatory function of Paupar. To investigate whether Paupar and KAP1 functionally interact to control gene expression, we first tested whether they regulate a common set of target genes. We depleted Kap1 expression in N2A cells using shRNA transfection and achieved ~90% reduction in both protein (Fig 2A) and transcript (Fig 2B) levels. Paupar levels do not change upon KAP1 knockdown, indicating that KAP1-dependent changes in gene expression are not due to regulation of Paupar expression (Fig 2B). Transcriptome profiling using microarrays then identified 1,913 differentially expressed genes whose expression significantly changed [at a 5% false discovery rate (FDR)] greater than 1.4-fold (log2 fold change = 0.5) upon KAP1 depletion (Fig 2C and Dataset EV2). 282 of these genes were previously identified to be regulated by human KAP1 in Ntera2 undifferentiated human neural progenitor cells (Iyengar et al., 2011). Transient reduction in KAP1 expression by ~55% using a second shRNA expression vector (Kap1 shB) also induced expression changes for seven out of eight KAP1 target genes with known functions in neuronal cells that were identified in the microarray (Fig EV2). These data further validate the specificity of the KAP1 regulated gene set.

We previously showed that Paupar knockdown induces changes in the expression of 942 genes in N2A cells (Vance et al., 2014). Examination of the intersection of KAP1 and Paupar transcriptional targets identified 244 genes whose levels are affected by both Paupar and KAP1 knockdown in this cell type (Fig 2D and Dataset EV3). This represents a significant 3.6-fold enrichment over the number expected by random sampling and is not due to co-regulation because Kap1 is not a Paupar target (Vance et al., 2014). A large majority (87%; 212/244) of these common targets are positively regulated by Paupar and for two-thirds of these genes (161/244) their expression changes in the same direction upon Paupar or KAP1 knockdown (Fig 2E). Furthermore, Gene Ontology enrichment analysis of these 244 genes showed that Paupar and KAP1 both regulate a shared set of target genes enriched for regulators of interphase, components of receptor tyrosine kinase signalling pathways as well as genes involved in nervous system development and essential neuronal cell functions such as synaptic transmission (Fig 2F). Genes targeted by both Paupar and KAP1 are thus expected to contribute to the control of neural stem cell self-renewal and neural differentiation.

**Paupar, KAP1 and PAX6 associate on chromatin within the regulatory region of shared target genes**

In order to investigate Paupar-mediated mechanisms of distal gene regulation, we next sought to determine whether Paupar, KAP1 and PAX6 can form a ternary complex on chromatin within the regulatory regions of their shared target genes. To do this, we first integrated our analysis of PAX6-regulated gene expression programmes in N2A cells (Vance et al., 2014) and identified 87 of the 244 Paupar and KAP1 common targets, which is 35.8-fold
greater than expected by random sampling, whose expression is also controlled by PAX6 (Fig 3A and Dataset EV3). We found that 34 of these genes contain a CHART-Seq mapped Paupar binding site within their GREAT defined putative regulatory regions (Vance et al., 2014; Vance, 2016) and predicted that these represent functional Paupar binding events within close genomic proximity to direct transcriptional target genes (Fig 3A and Dataset EV3).

ChIP-qPCR analysis previously identified four of these Paupar bound locations within the regulatory regions of the Mab21L2, Mst1, E2f2 and Igfbp5 genes that are also bound by PAX6 in N2A cells (Vance et al., 2014). We therefore measured KAP1 chromatin occupancy at these regions as well as at a negative control sequence within the first intron of E2f2 using ChIP and identified a specific enrichment of KAP1 chromatin association at the Mab21L2, Mst1, E2f2 and Igfbp5 genes compared to an IgG isotype control (Fig 3B). KAP1 binding to these regions is only two- to fourfold reduced compared to the Zfp382 3’UTR-positive control (Fig 3B), which represents an exemplar high-affinity KAP1 binding site (Iyengar et al., 2011). KAP1 and Paupar also co-occupy a binding site within the Ezh2 gene. Ezh2 is regulated by Paupar and KAP1 but not by PAX6 suggesting that transcription factors in addition to PAX6 may also be involved in modulating Paupar-KAP1 function. However, taken together these data indicate that Mab21L2, Mst1, E2f2 and Igfbp5 are co-ordinately regulated by a ribonucleoprotein complex containing Paupar-KAP1–PAX6.

Paupar functions as a transcriptional cofactor to promote KAP1 chromatin occupancy and H3K9me3 deposition at PAX6 bound sequences

KAP1 is recruited to its target sites within 3’UTRs of ZNF genes through association with KRAB-ZNF transcription factors (O’Geen
Figure 3.

**A** KAP1 Regulated Genes

```
| Genes   |
|---------|
| KAP1    |
| Paupar  |
```

**B** KAP1 Occupancy (% Input)

- **Mab21L2B5**
- **Mst1B5**
- **E2f2B5**
- **Igfbp5B5**
- **Ezh2B5**
- **Zip392**
- **Ezh2 Control**

![Graph showing KAP1 Occupancy](image)

**C** Relative expression

- **Paupar**

![Graph showing Relative expression](image)

**D** KAP1 Occupancy (% Input)

- **Scr**
- **sh408**
- **sh165**

![Graph showing KAP1 Occupancy](image)

**E** Paupar, KAP1, ACTIN

![Western Blot](image)

**F** IP: FLAG-PAX6

```
| IncRNA |
|--------|
| Paupar |
| Control|
```

![Western Blot](image)

**G** Relative expression

- **Paupar**

![Graph showing Relative expression](image)

**H** H3K9me3 (% Input)

- **Scr**
- **sh408**
- **sh1849**

![Graph showing H3K9me3](image)
et al, 2007; Iyengar et al, 2011). However, Paupar bound sequences are preferentially located at gene promoters and are not enriched for KRAB-ZNF transcription factor binding motifs as determined using de novo motif discovery (Vance et al, 2014). This suggests that Paupar may play a role in recruiting KAP1 to a separate class of binding sites in a KRAB-ZNF-independent manner. To test this, Paupar expression was first depleted using transient transfection of Paupar targeting shRNA expression vectors (Fig 3C). ChIP-qPCR was then performed to measure KAP1 chromatin occupancy in control and Paupar knockdown N2A cells at the four Paupar-KAP1-PAX6 co-occupied binding sites within the regulatory regions of the Mab21L2, Mst1, E2f2 and Igfbp5 genes, a Paupar-KAP1 bound sequence within the Ezh2 gene that is not regulated by PAX6, and a control sequence that is not bound by Paupar. The results show that KAP1 chromatin binding is significantly decreased at the four Paupar-KAP1-PAX6 bound regions upon Paupar depletion and that the extent of KAP1 chromatin association appears to be dependent on Paupar transcript levels (Fig 3D). KAP1 chromatin association is also not reduced at the Ezh2 gene Paupar-KAP1 binding site or at the control sequence that is not bound by Paupar (Fig 3D), whilst total KAP1 protein levels do not detectably change upon Paupar knockdown (Fig 3E), further confirming specificity.

These results imply that Paupar functions to promote KAP1 chromatin association at a subset of its genomic binding sites in trans and that this requires the formation of a DNA bound ternary complex containing Paupar, KAP1 and PAX6. Consistent with this, co-expression of Paupar promotes KAP1-PAX6 association in a dose-dependent manner in an immunoprecipitation experiment (Fig 3F). This effect is specific for the Paupar transcript because expression of a size-matched control RNA does not alter KAP1-PAX6 association. Paupar thus functions as a transcriptional cofactor to promote the assembly of a complex containing Paupar-KAP1-PAX6 on chromatin in trans. This ribonucleoprotein complex appears to function as a regulator of genes involved in controlling neural stem cell self-renewal and differentiation.

We next tested whether Paupar can induce histone modification changes at bound target genes on different chromosomes away from its sites of synthesis. As KAP1 interacts with the SETDB1 methyltransferase to mediate histone H3K9me3 deposition (Schultz et al, 2002), we first determined the levels of H3K9me3 at the shared binding sites near the Mab21L2, Mst1, E2f2, Igfbp5 and Ezh2 genes using ChIP-qPCR. This revealed an enrichment of H3K9me3-modified chromatin at all five locations (Fig EV3), consistent with a previous study showing that many KAP1 bound promoters are marked by H3K9me3 (O’Geen et al, 2007). ChIP analysis following Paupar depletion using two different shRNAs identified a significant decrease in histone H3K9me3 at three (Mab21L2, Mst1 and E2f2) out of four shared binding sites within genes that are co-regulated by Paupar, KAP1 and PAX6 (Fig 3G and H). No change in histone H3K9me3 was detected at the Ezh2 gene whose expression does not change upon PAX6 depletion. Together, these data show that Paupar functions to modulate KAP1 chromatin association and subsequent histone H3K9me3 deposition at a subset of its shared binding sites in trans.

Surprisingly, analysis of our microarray dataset of Paupar-mediated gene expression changes (Vance et al, 2014) showed that H3K9me3 deposition did not correlate with transcriptional repression. Mab21L2 and Mst1 were downregulated upon Paupar depletion suggesting that they are activated by the Paupar-KAP1-PAX6 complex whilst E2f2 expression was upregulated, suggesting that it is repressed by this complex. These findings are consistent with recent work using dCas9 fusion proteins to target histone methylation to specific loci (O’Geen et al, 2017) and suggest a complex relationship between Paupar-mediated KAP1-dependent chromatin changes and gene expression.

**Paupar co-occupies an enriched subset of KAP1 binding sites genome-wide**

We next examined the intersection between Paupar and KAP1 bound locations genome-wide in order to generate a more comprehensive view of the potential of Paupar for regulating KAP1 function. ChIP-seq profiling of KAP1 chromatin occupancy showed that KAP1 associates with 5,510 genomic locations compared to input DNA in N2A cells (1% FDR; Dataset EV4). KAP1 binding sites are particularly enriched at promoter regions, over gene bodies and at the 3’ UTRs of zinc finger genes (Fig 4A), consistent with previous studies mapping human KAP1 genomic occupancy (O’Geen et al, 2007; Iyengar et al, 2011). Intersection of KAP1 bound locations
Our results indicate that PAX6 is likely to play a regulatory role at a large proportion of Syt7 regulatory regions of four neuronal genes (binding sites close to the Mab21L2 and Kap1 sequences as well as at previously identified KAP1–PAX6 co-occupied sequences genome-wide).

We then examined the intersection between these 46 Paupar-KAP1 co-bound locations and the 244 Paupar-KAP1 co-regulated genes (Dataset EV3) and found shared binding sites within the putative regulatory regions of the Npy, Syt1, Fam92b and PlxnA4 genes. However, we expect this to be an under-representation of the total number of direct Paupar-KAP1 co-regulated targets given the complex cause-and-effect relationship between histone H3 methylation and gene expression (O’Geen et al., 2017).

Our analysis also revealed that only one of the 46 Paupar-KAP1 co-occupied sequences is located within the 3’ UTR of a ZNF gene (zfp68; Dataset EV4), pointing to an alternative mechanism of KAP1 genomic recruitment in addition to the well-described KRAB-ZNF association. To investigate this further, we performed ChIP-qPCR to interrogate the overlap between PAX6 and Paupar-KAP1 co-occupied locations. PAX6 occupancy was measured at a subset of ChIP-seq and CHART-seq defined KAP1-Pax6 bound sequences as well as at previously identified Paupar-KAP1–PAX6 binding sites close to the Mab21L2 and Mst1 genes as positive controls. The results identified statistically significant PAX6 enrichment at 11 out of 15 (73%) locations tested compared to an IgG control (Fig 4D). These include PAX6 binding sites within the regulatory regions of four neuronal genes (Npy, Syt1, Tshz2 and Syt7) whose expression changes when PAX6 expression is depleted in N2A cells (Vance et al., 2014). Taken together, these results indicate that PAX6 is likely to play a regulatory role at a large proportion of Paupar-KAP1 co-occupied sequences genome-wide. The absence of PAX6 from some of the tested locations further suggests that Paupar-KAP1 can associate with other transcription factors in addition to PAX6.

**Paupar and Kap1 regulate olfactory bulb neurogenesis**

Our results indicate that Paupar and Kap1 regulate the expression of shared target genes important for proliferation and neuronal differentiation in N2A cells. We next expanded this observation and tested whether Paupar and Kap1 can regulate mouse SVZ/OB neurogenesis in vivo. In this system, neurogenesis can be monitored by electroporating neonatal SVZ neural stem cells and analysing differentiated neurons that have migrated into the OB 7 days post electroporation (Boutin et al., 2008; Chesler et al., 2008). RT-qPCR first showed that Paupar and Kap1 are expressed in the SVZ and in neurospheres cultured from postnatal day 4 (P4) SVZ (Fig EV4A and B), consistent with Kap1 expression data in the Allen Brain Atlas. Similar to N2A cells, shRNA expression vectors depleted Paupar and Kap1 transcript in P4 SVZ neurospheres (Fig EV4C and D). Nucleofection of shRNA expression vectors targeted ~60% of cells, as measured using GFP, but we determined transcript levels in all cells. Thus, on a cell-by-cell basis the relative level of knockdown of both Paupar and Kap1 is predicted to be greater than shown.

We then electroporated P1 pups with Paupar and Kap1 shRNA expression constructs or a scrambled control and examined the number and morphology of neurons that migrated into the OB 7 dpe. The results showed that there were significantly fewer GFP+ cells in the OB after Paupar knockdown (KD) using sh165 KD compared to control whilst KD with sh408 caused a slight but statistically non-significant decrease in OB GFP+ cell numbers (Fig 5A and B). As sh165 more efficiently depletes Paupar expression compared to sh408 in N2A cells (Fig 3C) and in neurospheres (Fig EV4C), this result is suggestive of dose-dependent effects mediated by the Paupar transcript. Co-staining with the immature neuroblast marker DCX (Yang et al., 2004) showed that all GFP+ cells in the OB were DCX+ and this was not altered by Paupar KD (Fig EV4E). Similar to Paupar, at 7 dpe of either Kap1 shRNA expression construct, there was a significant reduction in the number of GFP+ cells that had migrated from the SVZ into the OB (Fig 5C and D). We controlled for apoptosis as this may lead to reduced cell numbers and did not detect changes in the percentage of GFP+ cells that are TUNEL+ at 3 or 7 dpe between scrambled control and any of the Paupar or Kap1 shRNA expression vectors in the SVZ, RMS or OB (Fig EV5). These results therefore suggest that both Paupar and Kap1 are required for the production of newborn OB neurons.

Interestingly, Paupar as well as Kap1 knockdown altered the morphology of newborn neurons in the OB (Fig 5E–H). As expected (Petretanu & Alvarez-Buylla, 2002), in scrambled controls many GFP+ neurons in the OB granule layer had begun morphological differentiation with processes extending radially towards the pial surface, some of which were branched. These cells were classified as class I (Fig 5E and F; Boutin et al., 2010). By contrast, after Paupar KD, a variety of abnormal morphologies...
Figure 4. Ioanna Pavlaki et al. Paupar functionally interacts with KAP1.
were observed, which we classified as class II or class III (Fig 5E). Class II cells were rare but were distinguished by many short branched processes. Class III cells exhibited only short or no processes, suggesting they were still migrating or had not substantially differentiated (Fig 5E). Quantification revealed that after electroporation the percentage of cells with Class I morphology was $34 \pm 2\%$ in scrambled controls but only $8 \pm 3\%$ after Paupar KD with sh165 and $6 \pm 3\%$ in the sh408 group ($P = 0.0005$ and $P = 0.0009$, respectively; Fig 5G). Conversely, after Paupar KD there were significantly more class III neurons in the sh165 group ($87 \pm 4\%$) as well as in the sh408 group ($85 \pm 6\%$) compared to controls ($58 \pm 5\%$; $P = 0.003$ and $P = 0.02$, respectively). Kap1 KD showed similar effects (Fig 5F and H); shA and shB resulted in $16.7 \pm 5.6$ and $19.3 \pm 2.0\%$ of Class I neurons versus $42.0 \pm 1.5\%$ in controls ($P = 0.012$ and $P = 0.013$, respectively). Again, the number of Class III neurons increased from $54.7 \pm 2.2\%$ in controls to $81.3 \pm 5.6\%$ after shA KD and $77.3 \pm 0.3\%$ after shB KD ($P = 0.0009$ and $P = 0.0005$, respectively). These data further suggest that Kap1 and Paupar affect postnatal neurogenesis by disrupting both migration into the OB and morphological differentiation of newborn neurons.

**Discussion**

LncRNAs can bind and regulate target genes on multiple chromosomes away from their sites of transcription. The number of IncRNAs that function in this way is steadily increasing suggesting that nuclear IncRNAs could exert a wide range of currently characterised, trans-acting functions in transcription and chromatin regulation. Moreover, loss-of-function studies using animal model systems are needed to identify and characterise lncRNA regulatory roles during embryonic development and in adult tissue homeostasis to clarify the importance of this class of transcript in vivo.

To gain novel insights into lncRNA gene regulation, we investigated the mode of action of the CNS-expressed lncRNA Paupar at chromosomal binding sites away from its site of synthesis in N2A cells. We show that Paupar directly binds the Kap1 epigenetic regulatory protein and thereby regulates the expression of shared target genes important for proliferation and neuronal differentiation. Our data indicate that Paupar modulates histone H3K9me deposition at a subset of distal bound transcriptional regulatory elements through its association with Kap1, including at a binding site upstream of the E2f2 gene. These chromatin changes are consistent with our previous report that this E2f2 bound sequence functions as a transcriptional enhancer whose activity is restricted by Paupar transcript levels (Vance et al., 2014). Our results therefore suggest a model in which Paupar-directed histone modification changes in trans alter the activity of bound regulatory elements in a dose-dependent manner.

Several other lncRNAs have also been shown to alter the chromatin structure of target genes in trans. These include the human Paupar orthologue which can inhibit H3K4 trimethylation of the Hes1 promoter in eye cancer cell lines, as well as IncRNA-HIT which induces p100/CBP-mediated changes in histone H3K27ac at bound sequences to regulate genes involved in chondrogenesis (Carlson et al., 2015; Ding et al., 2016). The lncRNA Hotair is one of the most studied trans-acting IncRNAs. Whilst Hotair has been proposed to guide PRC2 to specific locations in the genome to induce H3K27me3 and silence gene expression (Chu et al., 2011), recent conflicting studies report that PRC2 associates with low specificity to lncRNAs and suggest that HOTAIR does not directly recruit PRC2 to the genome to silence gene transcription (Kaneko et al., 2013; Davidovich et al., 2015; Portoso et al., 2017). Mechanistic studies on individual trans-acting lncRNAs such as Paupar are therefore needed to further define general principles of genome-wide lncRNA transcription and chromatin regulation.

It is proposed that lncRNAs may guide chromatin-modifying complexes to distal regions in the genome through RNA–DNA associations at transcribed loci, or either directly through RNA–DNA base pairing or indirectly through RNA–protein–DNA associations (Vance & Ponting, 2014; Rutenberg-Schoenberg et al., 2016). We show here that Paupar acts to increase Kap1 chromatin association by promoting the formation of a DNA binding regulatory complex containing Paupar, Kap1 and PAX6 within the regulatory regions of shared target genes in trans, as illustrated in the model in Fig 6. This suggests that Paupar functions as a cofactor for transcription factors such as PAX6 to modulate target gene expression across multiple chromosomes. In a similar manner, Prncre1 and Pegem1 IncRNAs interact with the androgen receptor (AR) and associate with non-DNA binding cofactors to facilitate AR-mediated gene regulation (Yang et al., 2013). LncRNA-mediated recruitment of chromatin regulatory proteins to DNA bound transcription factors may represent a common mechanism of trans-acting lncRNA gene regulation, in line with their suggested role as molecular scaffolds (Tsai et al., 2010).
Figure 5.

Ioanna Pavlaki et al. Paupar functionally interacts with KAP1.

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KAP1 is guided to the 3’ UTR of zinc finger genes in the genome through association with KRAB-ZNF transcription factors (O’Geen et al., 2007). However, the mechanisms of KAP1 genome-wide recruitment are not fully understood (Iyengar et al., 2011). Our data identify KAP1 as a novel RNA binding protein and show that Paupar plays a role in modulating the recruitment of KAP1 to specific PAX6 bound locations in the genome. We further assessed the extent to which Paupar may be able to modulate KAP1 genome-wide recruitment and identified 46 shared binding sites on chromatin, only one of which was within a 3’ UTR of a zinc finger gene. We measured PAX6 occupancy at a subset of these locations and identified significant PAX6 enrichment at 11 out of 15 Paupar-KAP1 co-occupied regions. These results raise the possibility that additional chromatin-enriched lncRNAs may operate to recruit KAP1 to specific locations in the genome and that this may involve context-specific interactions with both KRAB-ZNF and non-KRAB-ZNF containing transcription factors such as PAX6.

Our knockdown studies indicate that Paupar and Kap1 are required for normal postnatal OB neurogenesis in vivo. At 7 dpe of the SVZ most cells would be migrating from the RMS into the OB with only a minority differentiating and our quantification supported this. In accordance with the KAP1-Paupar physical association, both Paupar and Kap1 loss-of-function reduced the number of newborn neurons in the OB. Although the decrease in neuronal numbers could have been due to increased apoptosis caused by Paupar or KAP1 knockdown, we found no evidence of altered cell death in the SVZ, RMS or OB with TUNEL staining. Another possibility is that Paupar and KAP1 knockdown attenuated migration through the RMS. Reduced migration is sufficient to decrease rates of OB neurogenesis (Comte et al., 2011) and could explain the reduced number of cells in the OB 7 dpe. There is precedence for such an effect as reduction in either lncRNA ncRNA-7a or HNF1A-ASI decreased cell migration (Orom et al., 2010; Yang et al., 2014a), and reduced migration into the OB could also cause a delay in differentiation. Consistent with this, fewer newborn neuroblasts had differentiated morphology and more had immature morphology after Paupar or KAP1 depletion. Additionally, it may be that Paupar and KAP1 affect the rate of OB interneuron differentiation in a manner similar to NeuroD1 (Boutin et al., 2010; Pataskar et al., 2016).

This study identifies Paupar and Kap1 as novel regulators of OB neurogenesis in vivo and provides important conceptual insights into the distal modes of lncRNA-mediated gene regulation. Given the widespread role played by Kap1 in genome regulation and chromatin organisation, we anticipate that further chromatin-associated lncRNAs will be found to functionally interact with KAP1.

**Materials and Methods**

**Plasmid construction**

Kap1 and Paupar targeting short hairpin RNAs (shRNAs), designed using the Whitehead Institute siRNA selection programme, were synthesised as double-stranded DNA oligonucleotides and ligated...
into pBS-U6-CMVeGFP as shown previously (Vance et al., 2014). The Paupar targeting sh165 and sh408 expression constructs, the non-targeting scrambled control shRNA and pCAGGS-Paupar expression vector, are also detailed in (Vance et al., 2014). To generate the PAX6 expression vector, Pax6 coding sequence was PCR amplified from mouse N2A cell cDNA as a NotI-Xhol fragment and inserted into pcDNA3.1(+) (Invitrogen). The forward primer incorporated a DNA sequence to insert the DYKDDDDDK FLAG epitope tag in frame at the amino terminal end of PAX6. Rcor3 coding sequence was also PCR amplified from mouse N2A cell cDNA and cloned into pcDNA3.1(+) to generate pcDNA3-RCOR3. pcDNA3-HA-KAP1 was a kind gift from Colin Goding (Ludwig Institute, Oxford). The sequences of the oligonucleotides used in this study are listed in Table EV1.

**Cell culture**

N2A mouse neuroblastoma cells (ATCC CCL-131) were grown in DMEM supplemented with 10% foetal bovine serum. All transfections were performed using FuGENE 6 (Promega) following the manufacturer’s instructions. To generate Kap1 knockdown cells, ~2 × 10^5 cells were plated per well in a six-well plate. 16-24 h later, cells were transfected with 1.5 µg Kap1 shRNA expression construct and 300 ng (5:1 ratio) pTK-Hyg (Clontech). Three days after transfection, cells were trypsinised, resuspended in growth medium containing 200 µg/ml Hygromycin B and plated onto a 6-cm dish. Drug-resistant cells were grown for 7 days and harvested.

**Immunoprecipitation**

1 × 10^6 N2A cells were seeded per 10-cm dish. The next day, cells were transfected with different combinations of pcDNA3-FLAG-PAX6, pcDNA3-HA-KAP1, pcDNA3-RCOR3, pCAGGS-Paupar, pCAGGS-AK034351 control transcript or pcDNA3.1 empty vector. 6 µg plasmid DNA was transfected in total. Two days later, cells were washed twice with ice-cold PBS, transferred to 1.5 ml microcentrifuge tubes and lysed in 1 ml ice-cold IP Buffer (IPB; 50 mM Hepes pH 7.5, 350 mM NaCl, 1 mM MgCl2, 0.5 mM EDTA and 0.4% IGEPAL CA-630) for 30 min, 4°C with rotation. Lysates were pelleted at 16,000 g, 20 min, 4°C in a microfuge, supernatant was added to 30 µl anti-FLAG M2 Magnetic Beads (#M8823, Sigma) and incubated overnight at 4°C with rotation. Beads were washed three times with IPB and eluted in 20 µl Laemmli sample buffer for 5 min at 95°C. Bound proteins were detected by Western blotting using anti-FLAG M2 (F3165, Sigma), anti-KAP1 (ab10483, Abcam), anti-RCOR3 (A301-273A, Bethyl Laboratories) and Protein A HRP (ab7456, Abcam).

**RNA pulldown assay**

Sense RNA was in vitro-transcribed from pCR4-TOPO-Paupar using T7 RNA polymerase, according to manufacturer’s instructions (New England Biolabs). Transcribed RNA was concentrated and purified using the RNEasy MinElute Cleanup kit (Qiagen). Purified RNA was then 5’ end labelled with biotin-maleimide using a 5’ EndTag nucleic acid labelling system (Vector Laboratories). Streptavidin-coated Dynabeads M-280 (Invitrogen) were washed, prepared for RNA manipulation and the 5’ biotinylated RNA bound according to manufacturer’s instructions. N2A cell nuclear extract was diluted in affinity binding/washing buffer (150 mM NaCl, 50 mM HEPES, pH 8.0, 0.5% Igepal, 10 mM MgCl2) in the presence of 100 µg/ml tRNA, 40 U/ml RNaseOUT (Invitrogen) and a protease inhibitor cocktail (Roche). RNA-coated beads were incubated with nuclear extract at room temperature for 2 h with rotation. The supernatant was then removed, the beads washed six times (10 min) with affinity/washing buffer, and bound protein eluted by heating to 95°C in the presence of Laemmli sample buffer for 5 min. Samples were loaded onto a 10% Tris-glycine polyacrylamide gel (Bio-Rad) and subjected to denaturing SDS–PAGE until they just entered the resolving gel. Protein samples were then excised, diced and washed three times with nanopure water. Tryptic digest and mass spectrometry were performed by the Central Proteomics Facility (Dunn School of Pathology, University of Oxford).

**RNA-IP**

Approximately 1 × 10^7 N2A cells were used per RNA-IP. Native RNA-IP experiments were performed using the Magna RIP Kit (Millipore) according to the manufacturer’s instructions. UV-RIP was carried out as described in Vance et al (2014). We used the following rabbit polyclonal antibodies: anti-RCOR3 (A301-273A, Bethyl Laboratories), anti-CoREST (07-455, Millipore), anti-KAP1 (ab10483, Abcam), anti-ERH (ab96130, Abcam), anti-PPAN (11006-1-AP, Proteintech Group) and rabbit IgG (PP64B, Millipore).

**Chromatin immunoprecipitation**

For knockdown experiments, 4 × 10^6 N2A cells per ChIP were seeded in 15-cm plates. The next day, cells were transfected with either 15 µg Paupar targeting shRNA expression vectors or a non-targeting scr control. Three days later, cells were harvested for ChIP using either 5 µg anti-KAP1 (ab10483, Abcam), anti-histone H3K9me3 (93161, Active Motif) or normal rabbit control IgG (#2729, Cell Signalling Technology) antibodies. ChIP was performed as described in Vance et al (2014). 5 µg anti-PAX6 (#AB2237, Millipore) was used for PAX6 ChIP. For KAP1 ChIP-seq, the following modifications were made to the protocol: ~2 × 10^7 N2A cells per ChIP were double-cross-linked, first using 2 mM disuccinimidyl glutarate (DSG) for 45 min at room temperature, followed by 1% formaldehyde for 15 min at room temperature, as described in Nowak et al (2005). Chromatin was sheared to ~200 bp using a Bioruptor Pico (Diagenode) and ChIP DNA and matched input DNA from two independent KAP1 ChIP experiments were sequenced on an Illumina HiSeq 4000 (150-bp paired-end sequencing).

**ChIP-seq analysis**

The Babraham Bioinformatics fastqscreen (https://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/) and fastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) tools were used to screen the raw reads for containments and to assess quality. We removed traces of the adapter sequence from the raw reads using the Trimmomatic tool (Bolger et al, 2014). Trimmomatic was also used to trim by quality with the options: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:50. The trimmed reads were then mapped to the mouse genome using BWA MEM (Li and Durbin, 2009).
were aligned to the mm10 reference genome, using the Burrows-Wheeler Aligner (Li & Durbin, 2010) with the command: > bwa mem mm10 < pair_1.fq > < pair_2.fq >. Alignment quality was assessed with the Qualimap 2.2.1 tool (Okonechnikov et al., 2016). The aligned reads were filtered to exclude reads with a MAPQ alignment quality < 20. Furthermore, we excluded reads aligning to blacklisted regions identified by the ENCODE consortium (ENCODE Project Consortium, 2012). MACS2 version 2.1.1.20160309 was used to identify genomic regions bound by KAP1. We further filtered the aligned reads to retain only those with length 150 and called peaks relative to the input controls using the options “–gsise = 1.87e9 – qvalue = 0.01 -B –keep-dup auto”. To examine the read density distribution in the vicinity of KAP1 peaks, we used deepTools (Ramirez et al., 2016). Read density was calculated with respect to input using the bamCompare tool from deepTools, with the option “–binSize 10”. The matrix of read densities in the vicinity of KAP1 peaks was calculated using “computeMatrix reference-point”, and heatmaps plotted with “plotHeatmap”. The Genomic Association Test tool GAT (Heger et al., 2013) was used to characterise KAP1 binding sites and the relationship between KAP1 and Paupar. Coordinates with respect to the mm10 reference genome for characteristic genomic regions (exons, introns, 3′ UTRs, etc.) were downloaded from the UCSC Genome Table Browser (https://genome.ucsc.edu/cgi-bin/hgTables). The enrichment of KAP1 peaks and the intersection of KAP1 and Paupar peaks with respect to these genomic regions was assessed using GAT with the options “–ignore-segment-track –num-samples = 100,000” and using the complement of the blacklist regions as the workspace. To test for significance coincidence of KAP1 and Paupar peaks, we use GAT with the same options. The Paupar CHART-Seq peakset from Vance et al. (2014) was used for comparison.

**Transcriptomic analysis**

Total RNA was isolated from triplicate control and KAP1 knockdown cells using the Qiagen Mini RNeasy kit following the manufacturer’s instructions. RNA samples with a RNA Integrity Number greater than 8, as assessed on a BioAnalyzer (Agilent Technologies), were hybridised to Mouse Gene 1.0 ST Arrays as detailed in (Chalei et al., 2014). Microarray data were Robust Multi-array Average (RMA) normalised using GeneSpring GX12.6 (Agilent). Differentially expressed genes (fold change difference ≥ 1.4) were identified using a false discovery rate of ≤ 0.05 with a Benjamini and Hochberg multiple testing correction (Limma). Gene Ontology analysis was performed as previously (Vance et al., 2014).

**Neurosphere assay**

Neurospheres were cultured according to standard protocols as previously described (Dizon et al., 2006). In brief, age P3-P6 CD1 mice pups were anesthetised with hypothermia and decapitated, and the brains were immediately dissected out and sectioned in the coronal plane with a Meltwain tissue chopper. The SVZ was then dissected out in ice-cold HBSS in a sterile laminar flow hood. Accutase was used for 15 min for cell dissociation. Cells were cultured in defined Neurobasal media supplemented with 20 ng/ml EGF (Sigma) and 20 ng/ml bFGF (R&D). Cells were seeded at a density of 100 cells/µl and passaged every 3–4 days.

**Neural stem cell nucleofection**

3–4 × 10⁶ dissociated neurosphere cells were nucleofected according to the protocol of LONZA (VPG-1004). Cells were mixed with 100 µl nucleofection solution (82 µl of Nucleofector Solution + 18 µl of supplement) and 5–10 µg DNA and transferred into cuvettes. 500 µl of culture medium was added into the cuvette, and the sample was then transferred into 1 ml medium and centrifuged at 250 g for 5 min. Cells were resuspended with fresh medium and plated at 200,000 cells/ml in a PolyHeme-coated 6-well plate.

**Postnatal electroporation**

Electroporation was performed as published (Boutin et al., 2008; Chesler et al., 2008). DNA plasmids were prepared with Endofree Maxi kit (Qiagen) and mixed with 0.1% fast green for tracing. DNA concentrations were matched in every individual experiment. P1 CD1 pups were anesthetised with hypothermia, and 1–2 µl of plasmid was injected with glass capillary. Electrical pulses (100 V, 50 ms ON with 850-ms intervals for five cycles) were given with tweezer electrodes (CUY650PS). Pups were recovered, then returned to dam and analysed at the indicated time.

**Immunohistochemistry and imaging**

Immunohistochemistry was as previously described (Young et al., 2014) using Chicken anti-GFP (1:500, Aves) and goat anti-DCX (1:100, Santa Cruz) primary and Alexafluor-conjugated (Invitrogen) secondary antibodies. TUNEL method was performed using the In situ cell death detection kit, TMR red (Roche-12156792910) to detect apoptosis. Sections were imaged with Zeiss 710 Laser Scanning Microscopy. For co-localisation in GFP+ cells, a 20 × oil immersion objective was used and Z stacks were generated at 2-µm intervals. Confocal images were analysed with ImageJ.

**Morphological evaluation**

All GFP+ neuroblasts in the granule layer of the OB were binned into Class I, II or III groups similar to a previous study (Boutin et al., 2010). Only cells with obvious cell bodies and that were entirely found in the field were included. Cells in the rostral migratory stream in the core of the OB, and in OB layers outside of the granule layer, were not included. N = 3–5 mice per group.

**Ethics**

All mouse experiments were performed in accordance with institutional and national guidelines and regulations under UK Home Office Project Licence PPL 3003311.

**Data availability**

The ChIP-Seq and microarray data have been deposited in the GEO database https://www.ncbi.nlm.nih.gov/geo/ under the following accession numbers: GSE110032 and GSE110033.

**Expanded View** for this article is available online.
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Author contributions

KWW conceived the study. IP, FA, BS, TS, SL, FGS and KWV analysed and interpreted the data. NC, SL and DJW carried out computational analysis of the microarray and ChIP-seq data. KWW and FGS wrote the manuscript with input from IP and CPP who reviewed and edited the drafts. CPP, FGS and KWV supervised the research and acquired funding.

Conflict of interest

The authors declare that they have no conflict of interest.

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