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The role of long non-coding RNAs (lncRNAs) in mammalian brain development

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ELPINICKIE NINOU, BIOLOGIST

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Τμήμα Βιολογίας

Ο ρόλος των μακρών RNAs που δεν κωδικοποιούν για πρωτεΐνες (lncRNAs) στην ανάπτυξη του εγκεφάλου των θηλαστικών

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

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This PhD Thesis was performed in Dr. Panagiotis Politis' Laboratory, Center of Basic Research, Biomedical Research Foundation Academy of Athens.

Three – member advisory committee:

1. George Diallinas, PhD, Professor of Molecular Microbiology, NKUA
2. Spiros Efthimiopoulos, PhD, Professor of Animal & Human Physiology – Neurobiology, NKUA
3. Panagiotis Politis, PhD, Investigator – Associate Professor Level, Center Basic Research, BRFAA

Seven – member board of examination:

1. George Diallinas, PhD, Professor of Molecular Microbiology, NKUA
2. Spiros Efthimiopoulos, PhD, Professor of Animal & Human Physiology – Neurobiology, NKUA
3. Panagiotis Politis, PhD, Investigator – Associate Professor Level, Center Basic Research, BRFAA
4. Panagiota Papazafiri, PhD, Associate Professor of Animal Physiology, NKUA
5. Dimitra Thomaidou, PhD, Research Director, Laboratory of Cellular & Molecular Neurobioloby, Institute Pasteur Hellenique
6. Evangelia Emmanouilidou, PhD, Assistant Professor of Biochemistry, NKUA
7. Ioannis Serafimidis, PhD, Investigator – Assistant Professor Level, BRFAA

Summary

With the advent of new generation sequencing technologies, a growing list of formerly unknown regulatory RNA species have come into spotlight. Among them, long non-coding RNAs (lncRNAs) have been found to control stem cell pluripotency, carcinogenesis, development and function of several tissues and organs. Although thousands of lncRNAs are expressed in adult mammalian brain in a highly patterned and specific manner, they remain poorly characterized and their roles in brain development have not yet been studied.

To tackle this question, we initially performed RNA-Seq analysis in the developing nervous system of mouse embryo at embryonic day E12.5. Based on this analysis, we identified many lncRNAs highly expressed in neural cells. We focused on lncRNAs, which are transcribed from genomic loci in close proximity with protein coding genes, encoding for critical transcription factors (TFs) in brain development. We hypothesized that these lncRNAs may be implicated in the regulation of neighboring TF genes.

To this end, we characterized the changes in the expression profiles of a number of lncRNAs-TF pairs during development of mouse brain with real time-qPCR and *in situ* hybridizations. In this study, we focused firstly on the functional role of lncRNA TCONS_00034309, named by us as *Lacuna*, in the differentiation of neural stem cells and its relation to *Tbr2* transcription factor, a critical regulator of neurogenesis, by *ex vivo* overexpression and knockdown studies.

More specifically, *Lacuna* gene is on chromosome 9, around 1kb away from *Tbr2/Eomes* gene. Its transcript consists of 3 exons and a total length of 1661 nt, as confirmed by our mapping strategy. *Lacuna* is differentially expressed in the developing mouse brain with higher expression during embryonic days E15 and E16. *In situ* hybridizations showed specific expression in the ventricular zone and cortical plate of the developing mouse cortex. Subcellular fractionation of neural stem cells and subsequent real time-qPCR revealed that *Lacuna* is found both in the cytosol and the nucleus, suggesting the possibility that it functions both *in cis* (affecting the neighboring gene) and *in trans* (affecting distal gene/genes or being involved in cytoplasmic processes).

To further clarify the functional role of *Lacuna*, we used a culture system for primary neural stem/progenitor cells, where progenitor/stem cells are isolated from mouse embryonic cortices of E14.5 and then, cultured appropriately to form neurospheres. In the presence of growth factors, neural stem cells (NSCs) are proliferating, whereas in the absence of growth factors, NSCs are differentiating into neurons and astrocytes. Using this culture system and upon overexpression of *Lacuna*, neurogenesis is

significantly reduced (b-III tubulin and NeuN markers) and Olig2+ population is increased. On the other hand, astrogliogenesis doesn't seem affected, as well as proliferation (BrdU+ index) and apoptosis (cleaved caspase 3+ index), but Nestin, a marker of neural cell stemness, is increased. Moreover, TBR2/EOMES+ population and *Tbr2/Eomes* expression are not affected by *Lacuna* overexpression, indicating that the effect on neurogenetic events is *Tbr2* independent and suggesting a possible *in trans* action of *Lacuna* lncRNA.

In order to further elucidate this, we performed knock-down experiments in the same culture system, using a CRISPR-dCas9-KRAB Effector System to repress the transcription of *Lacuna*. After confirming the effectiveness of the technique (*Lacuna* is significantly repressed), we also confirmed that the system does not affect the locus in general (the neighboring genes *Golga4* and *Gm33460* are unaffected). Of note, knockdown of *Lacuna* in NSCs in the presence of growth factors results in dramatic downregulation of *Tbr2/Eomes* gene, suggesting that *Lacuna* is necessary for *Tbr2* expression in NSCs.

Furthermore, in the absence of growth factors, *Lacuna* knockdown significantly promotes the differentiation of NSCs into both neurons (b-III tubulin+ index and NeuN+ index) and astrocytes (GFAP+ index), whereas the *Olig2+* progenitor population and the Nestin+ cells are decreased. In this setup, *Tbr2* is not expressed, as most of the cells have already been committed to a cell fate, suggesting that *Lacuna* has an *in trans*, differentiation-inhibiting action in NSCs.

Another lncRNA that drew our attention is *Lockd*, a lncRNA that was already studied in an erythroid cell line. *Lockd* is a 434 nt lncRNA located 4 kb away from *Cdkn1b* gene. *Cdkn1b* encodes for p27, a well studied cyclin-dependent kinase inhibitor. p27 is extensively studied in the nervous system, with established roles in promoting cell cycle exit, neuronal differentiation and migration. Intriguingly, both *Lockd* and p27 are differentially expressed during mouse brain development, but their expression profiles are opposite. Due to the proximity of *Lockd* and p27 loci and the involvement of p27 in cell cycle exit events, we first used N2A cells, a fast-growing mouse neuroblastoma cell line, to study *Lockd* and its relation to p27.

Indeed, upon overexpression of *Lockd* lncRNA in N2A cells, proliferation is significantly increased. Furthermore, under the same conditions, p27 expression is repressed, proposing that *Lockd* inhibits expression of p27, which in turn results in increased proliferation, as p27 physiologically promotes cell cycle exiting. As proliferation is a crucial process in brain development and neural stem cells, we also examined *Lockd* expression in NSCs cultures.

In fact, *Lockd* is expressed in NSC cultures, but more interestingly, it is significantly downregulated in minus growth factors conditions in comparison to plus growth factors conditions. Additionally, upon overexpression of *Lockd* in NSCs, proliferation is increased and p27 expression is repressed, as in N2A cells. These primary findings reveal an exciting relation of *Lockd* with p27, but also an important role of this lncRNA in the proliferation of N2A cells and neural stem cells.

To conclude, our data suggest that lncRNAs are new key players in differentiation and proliferation during brain development and we provided at least two such examples. *Lacuna*, a novel lncRNA, is necessary for *Tbr2* expression and inhibits differentiation of NSCs and *Lockd*, an already studied lncRNA in another system, affects negatively p27 expression and promotes proliferation. Our study provides insights into the involvement of lncRNAs in organogenesis of the CNS and shows that lncRNAs and protein-coding genes form regulatory networks with important functions in neural stem cells and brain development.

Περίληψη

Με την έλευση και διάδοση των τεχνολογιών αλληλούχισης νέας γενιάς, ένας αυξανόμενος αριθμός πρώην άγνωστων ρυθμιστικών ειδών RNA έχει έρθει στο προσκήνιο. Μεταξύ αυτών, βρέθηκαν μακρά RNA μόρια που δεν κωδικοποιούν για πρωτεΐνες (long non-coding RNAs – lncRNAs), τα οποία συμμετέχουν στον έλεγχο της πολυδυναμίας των βλαστικών κυττάρων, την καρκινογένεση, την ανάπτυξη και τη λειτουργία πολλών ιστών και οργάνων. Παρόλο που χιλιάδες lncRNAs εκφράζονται στον εγκέφαλο των ενήλικων θηλαστικών με ειδικά και συγκεκριμένα μοτίβα έκφρασης, παραμένουν ελάχιστα αναγνωρισμένα και χαρακτηρισμένα, ενώ οι ρόλοι τους στην ανάπτυξη του εγκεφάλου δεν έχουν ακόμη μελετηθεί.

Η αντίληψη ότι το γονιδίωμα ασκεί τις λειτουργίες του μόνο μέσω πρωτεϊνών και τυπικών γονιδίων που κωδικοποιούν πρωτεΐνες εμφανίζεται προοδευτικά ως μια μάλλον αφελής απλοποίηση ενός σύνθετου και γοητευτικού συστήματος που βρίθει βρόγχων και δικτύων και που περιλαμβάνει επίσης μόρια RNA εκτός από πρωτεΐνες. Πράγματι, η γνώση μας για τη δραστηριότητα, τη ρύθμιση και την αρχιτεκτονική των γονιδιωμάτων των θηλαστικών έχει αναβαθμιστεί πλήρως μετά από τις τελευταίες εξελίξεις στις τεχνολογίες αλληλούχισης και στα δεδομένα που αποκτήθηκαν από μελέτες μεγάλης κλίμακας όπως το ENCODE και το FANTOM. Επιπλέον, οι συγκρίσεις μεταξύ μεταγραφωμάτων και γονιδιωμάτων θηλαστικών έχουν δείξει ότι περίπου τα δύο τρίτα του γονιδιωματικού DNA μεταγράφονται, αλλά μόνο λιγότερο από το 2% μεταφράζεται τελικά σε πρωτεΐνες. Επιπλέον, ακόμη και αν ληφθούν υπόψη τα παράγωγα που προκύπτουν από εναλλακτικό μάτισμα και από μετα-μεταγραφικές τροποποιήσεις, το επίπεδο της πολυπλοκότητας των οργανισμών μεταξύ των ευκαρυωτικών συσχετίζεται καλύτερα με το κλάσμα των RNAs που δεν κωδικοποιούν για πρωτεΐνες παρά με το άθροισμα των γονιδίων που κωδικοποιούν για πρωτεΐνες.

Εκτός από την ήδη γνωστή πληθώρα και πολυπλοκότητα που επιτυγχάνεται από τα γονίδια που κωδικοποιούν για πρωτεΐνες, τα ρυθμιστικά τους στοιχεία και τις μεταξύ τους αλληλεπιδράσεις, έχει αναγνωριστεί ένας εκπληκτικός αριθμός μη κωδικοποιητικών RNA (ncRNAs). Αυτά τα ncRNAs ταξινομούνται σε μικρά (scnRNAs) και μακριά (lncRNAs) μη κωδικοποιητικά RNA, τα οποία διαφέρουν κυρίως σε μέγεθος, αλλά και σε λειτουργία. Τα περισσότερα από τα sncRNA λειτουργούν ως ρυθμιστές σε μετα-μεταγραφικό επίπεδο στο κυτταρόπλασμα, ενώ τα lncRNAs δρουν σε ένα πιο ευρύ και ποικίλο εύρος λειτουργιών. Στην πραγματικότητα, διαπιστώθηκε ότι οι ρυθμιστικοί μηχανισμοί που περιλαμβάνουν lncRNAs επηρεάζουν εκτενώς τη γονιδιακή ρύθμιση μεμονωμένων γονιδίων και γονιδιακών δικτύων σε μεταγραφικό, μετα-μεταγραφικό και μεταφραστικό επίπεδο. Επομένως, τα

lncRNAs παρέχουν στα κύτταρα ένα επιπλέον εργαλείο για τον έλεγχο της χωροχρονικής ρύθμισης των γονιδίων, μια κρίσιμη απαίτηση για τα νευρικά βλαστικά κύτταρα κατά την ανάπτυξη του εγκεφάλου.

Ο κύριος στόχος αυτής της διδακτορικής διατριβής είναι η καλύτερη κατανόηση της εμπλοκής των lncRNAs στην ανάπτυξη του εγκεφάλου των θηλαστικών και ειδικά στα γονιδιακά ρυθμιστικά δίκτυα που ορίζουν την ταυτότητα των νευρικών βλαστικών κυττάρων. Για το σκοπό αυτό, οι συγκεκριμένοι στόχοι αυτής της διατριβής περιλαμβάνουν:

α) Ταυτοποίηση lncRNAs που εκφράζονται σε νευρικά κύτταρα και χαρακτηρισμός των αλλαγών στο προφίλ έκφρασης αυτών των lncRNAs κατά την ανάπτυξη εγκεφάλου μυός (και πιο συγκεκριμένα του τελεγκεφάλου).

β) Αξιολόγηση της σημαντικότητας των lncRNAs που προσδιορίστηκαν στον προηγούμενο στόχο και συγκεκριμένα αν απαιτούνται για την ανάπτυξη του εγκεφάλου μυός. Εδώ σκοπεύουμε να εξετάσουμε με συνολικό τρόπο το ρόλο των lncRNAs που προέκυψαν από τον προηγούμενο στόχο στον έλεγχο των ιδιοτήτων των νευρικών βλαστικών κυττάρων μυός. Έχουμε αναπτύξει πειραματικά συστήματα για την απομόνωση, την καλλιέργεια και τη διαφοροποίηση των νευρικών βλαστικών κυττάρων από το εμβρυϊκό κεντρικό νευρικό σύστημα μυών και έχουμε χρησιμοποιήσει αυτά τα συστήματα για να μελετήσουμε τους μοριακούς μηχανισμούς που ελέγχουν τον πολλαπλασιασμό, την επιβίωση, την κυτταρική εξειδίκευση και τη διαφοροποίηση των νευρικών βλαστικών κυττάρων. Αυτός ο στόχος περιλαμβάνει δύο συγκεκριμένους υπο - στόχους:

1: Υπερέκφραση επιλεγμένων lncRNAs και ανάλυση της επίδρασης της υπερέκφρασής τους σε πρωτογενείς καλλιέργειες νευρικών βλαστικών κυττάρων.

2: Αποσιώπηση επιλεγμένων lncRNAs χρησιμοποιώντας ένα σύστημα CRISPR-dCas9-KRAB και ανάλυση της επίδρασης της αποσιώπησής τους σε πρωτογενείς καλλιέργειες νευρικών βλαστικών κυττάρων.

Ο απώτερος στόχος αυτής της διατριβής είναι να παρέχει πληροφορίες για τη συμμετοχή των lncRNAs στην οργανογένεση και να συμβάλλει στην κατανόηση του τρόπου με τον οποίο τα lncRNAs και τα γονίδια που κωδικοποιούν για πρωτεΐνες σχηματίζουν ρυθμιστικά δίκτυα με σημαντικές λειτουργίες στα νευρικά κύτταρα. Η κατανόηση του ρόλου των lncRNAs στην οργανογένεση του εγκεφάλου θα μπορούσε να επιφέρει σημαντικότατες προσθήκες στις βασικές αρχές της αναπτυξιακής / κυτταρικής βιολογίας και της νευροεπιστήμης.

Για να αντιμετωπίσουμε λοιπόν το βασικό μας ερώτημα, αρχικά πραγματοποιήσαμε RNA-Seq ανάλυση στο αναπτυσσόμενο νευρικό σύστημα εμβρύων μυός. Με βάση αυτήν την ανάλυση, εντοπίσαμε πολλά lncRNAs με υψηλά επίπεδα γονιδιακής έκφρασης σε κύτταρα του νευρικού συστήματος. Εστιάσαμε σε lncRNAs, τα οποία μεταγράφονται από γονιδιωματικούς τόπους σε κοντινή απόσταση με γονίδια που κωδικοποιούν για πρωτεΐνες και πιο συγκεκριμένα, σε κοντινή απόσταση με γονίδια που κωδικοποιούν για μεταγραφικούς παράγοντες με κρίσιμο ρόλο στην ανάπτυξη του εγκεφάλου. Βασιζόμενοι και στη σχετική βιβλιογραφία που πρότεινε ως πιθανό μηχανισμό δράσης των lncRNAs την in cis επίδρασή τους σε διπλανά γονίδια, υποθέσαμε ότι αυτά τα lncRNAs μπορεί να εμπλέκονται στη ρύθμιση των γειτονικών τους γονιδίων που κωδικοποιούν για σημαντικούς μεταγραφικούς παράγοντες.

Για να ελέγξουμε αυτήν την αρχική μας υπόθεση, μελετήσαμε τις αλλαγές στα προφίλ έκφρασης κατά την ανάπτυξη του εγκεφάλου μυός ενός αριθμού ζευγαριών lncRNAs και γειτονικών γονιδίων για μεταγραφικούς παράγοντες με real time-qPCR. Από αυτήν τη μελέτη, προέκυψαν ενδιαφέροντα τέτοια ζεύγη, τα οποία αναλύσαμε περαιτέρω με in situ υβριδισμούς σε ιστούς διάφορων αναπτυξιακών σταδίων εμβρυικού εγκεφάλου μυός και κατόπιν υπερέκφρασης των εν λόγω lncRNAs στην κυτταρική σειρά νευροβλαστώματος N2A. Αυτά τα πειράματα, έδωσαν αρκετές πληροφορίες, ώστε αρχικά να εστιάσουμε το ενδιαφέρον μας στο lncRNA TCONS_00034309, που ονομάστηκε από εμάς ως Lacuna. Επομένως, συνεχίσαμε με ex vivo μελέτες υπερέκφρασης και αποσιώπησης του Lacuna σε νευρικά βλαστικά κύτταρα, ώστε να μελετήσουμε το λειτουργικό ρόλο του στη διαφοροποίηση των νευρικών βλαστικών κυττάρων και τη σχέση του με το μεταγραφικό παράγοντα Tbr2 ή Eomes, έναν κρίσιμο ρυθμιστή της νευρογένεσης και συγκεκριμένα της μετάβασης από ενδιάμεσο προγονικό κύτταρο σε μετα-μιτωτικό νευρώνα.

Πιο συγκεκριμένα, το γονίδιο που κωδικοποιεί για το Lacuna βρίσκεται στο χρωμόσωμα 9, μόνο περίπου 1kb μακριά από το γονίδιο που κωδικοποιεί για το μεταγραφικό παράγοντα Tbr2 / Eomes. Το μετάγραφο αποτελείται από 3 εξώνια και έχει συνολικό μήκος 1661 νουκλεοτιδίων, όπως επιβεβαιώνεται από τη στρατηγική χαρτογράφησης που ακολουθήσαμε. Το Lacuna εκφράζεται διαφορικά στον αναπτυσσόμενο εγκέφαλο μυός με υψηλότερη έκφραση κατά τη διάρκεια των εμβρυϊκών ημερών E15 και E16. Οι in situ υβριδισμοί που πραγματοποιήσαμε για το Lacuna σε ιστούς εμβρυικού εγκεφάλου μυός έδειξαν ειδική έκφραση στην κοιλιακή στιβάδα και στη φλοιική πλάκα του αναπτυσσόμενου φλοιού εγκεφάλου μυός. Η υποκυτταρική κλασμάτωση των νευρικών βλαστικών

κυττάρων και η επακόλουθη real time-qPCR αποκάλυψαν ότι το Lacuna βρίσκεται τόσο στο κυτοσόλιο όσο και στον πυρήνα, υποδηλώνοντας την πιθανότητα να λειτουργεί τόσο in cis όσο και in trans.

Για να αποσαφηνίσουμε περαιτέρω το λειτουργικό ρόλο του Lacuna, χρησιμοποιήσαμε ένα σύστημα καλλιέργειας για πρωτογενή νευρικά βλαστικά / προγονικά κύτταρα, όπου τα προγονικά / βλαστικά κύτταρα απομονώνονται από φλοιούς εμβρυικών εγκεφάλων μυός κατά την εμβρυική ημέρα E14.5 και στη συνέχεια, καλλιεργούνται κατάλληλα για να σχηματίσουν νευροσφαίρες. Παρουσία αυξητικών παραγόντων, τα νευρικά βλαστικά κύτταρα πολλαπλασιάζονται, ενώ απουσία αυξητικών παραγόντων, διαφοροποιούνται σε νευρώνες και αστροκύτταρα. Χρησιμοποιώντας αυτό το σύστημα καλλιέργειας και μετά από υπερέκφραση του lncRNA Lacuna και ανοσοφθορισμούς, είδαμε ότι η νευρογένεση μειώνεται σημαντικά (δείκτες β-III τουμπουλίνη και NeuN) και ο πληθυσμός Olig2+ αυξάνεται στα κύτταρα που υπερεκφράζουν το Lacuna σε σύγκριση με τις καλλιέργειες ελέγχου. Από την άλλη πλευρά, η αστρογλοιογένεση δεν φαίνεται να επηρεάζεται, καθώς και ο πολλαπλασιασμός (δείκτης BrdU +) και η απόπτωση (δείκτης διασπασμένης κασπάσης 3), αλλά η νεστίνη, ένας δείκτης της πολυδυναμίας των νευρικών βλαστικών κυττάρων, αυξάνεται. Επιπλέον, ο πληθυσμός των κυττάρων που είναι TBR2 / EOMES + και η γονιδιακή έκφραση του παράγοντα Tbr2 / Eomes δεν επηρεάζονται από την υπερέκφραση του Lacuna, γεγονός που υποδηλώνει ότι η επίδραση του Lacuna στις διαδικασίες της νευρογένεσης είναι ανεξάρτητη από το Tbr2 και υπαινίσσεται μια πιθανή in trans δράση του lncRNA Lacuna.

Για να διαλευκάνουμε περαιτέρω το ρόλο του Lacuna στη διαφοροποίηση των νευρικών βλαστικών κυττάρων, πραγματοποιήσαμε πειράματα αποσιώπησης του Lacuna στο ίδιο σύστημα καλλιέργειας, χρησιμοποιώντας ένα CRISPR-dCas9-KRAB Effector Σύστημα για την καταστολή της μεταγραφής του Lacuna. Αυτό το σύστημα είναι πολύ αποτελεσματικό στην αποσιώπηση γονιδίων που κωδικοποιούν για lncRNAs και επιλύει το ζήτημα αν τα παρατηρούμενα φαινόμενα αυτών των αποσιωπήσεων είναι αποτέλεσμα της απουσίας του lncRNA μορίου ή του αντίστοιχου DNA. Ο μηχανισμός δράσης αυτού του συστήματος δεν επηρεάζει καθόλου την DNA αλληλουχία. Η dCas9 μαζί με το μεταγραφικό καταστολέα KRAB οδηγούνται στο γονίδιο επιλογής από ειδικά σχεδιασμένα gRNA μόρια και έτσι επιτυγχάνεται η καταστολή της μεταγραφής του γονιδίου επιλογής. Αφού επιβεβαιώσαμε την αποτελεσματικότητα της τεχνικής (το Lacuna καταστέλλεται σημαντικά), επαληθεύσαμε επίσης ότι το σύστημα δεν επηρεάζει γενικά το γενετικό τόπο (τα γειτονικά γονίδια Golga4 και Gm33460 δεν επηρεάζονται). Αντιθέτως, η αποσιώπηση του Lacuna σε νευρικά βλαστικά κύτταρα παρουσία αυξητικών παραγόντων οδηγεί σε δραματική μείωση της έκφρασης του γονιδίου Tbr2 / Eomes, γεγονός που

υποδηλώνει ότι το Lacuna είναι απαραίτητο για την έκφραση του Tbr2 / Eomes σε νευρικά βλαστικά κύτταρα.

Επιπλέον, απουσία αυξητικών παραγόντων, η αποσιώπηση του Lacuna προάγει σημαντικά τη διαφοροποίηση των νευρικών βλαστικών κυττάρων σε μετα-μιτωτικούς νευρώνες (δείκτης β-III τουμπουλίνης και δείκτης NeuN) και σε αστροκύτταρα (δείκτης GFAP), ενώ ο πληθυσμός των προγονικών κυττάρων που είναι Olig2 + και ο πληθυσμός των βλαστικών κυττάρων που είναι νεστίνη+ μειώνονται. Σε αυτές τις συνθήκες, όπου τα νευρικά βλαστικά κύτταρα έχουν καλλιεργηθεί απουσία αυξητικών παραγόντων, ο μεταγραφικός παράγοντας Tbr2 / Eomes δεν εκφράζεται, καθώς τα περισσότερα από τα κύτταρα έχουν ήδη επιλέξει κυτταρική μοίρα ή απλούστερα, έχουν ξεκινήσει τη διαφοροποίησή τους, υποδηλώνοντας ότι το Lacuna έχει μια δράση in trans που αναστέλλει τη διαφοροποίηση των νευρικών βλαστικών κυττάρων.

Ένα άλλο lncRNA που προσέλκυσε την προσοχή μας είναι το Lockd, ένα lncRNA που έχει ήδη μελετηθεί σε μια κυτταρική σειρά ερυθροκυττάρων. Το Lockd είναι ένα lncRNA αποτελούμενο από 434 νουκλεοτίδια και βρίσκεται περίπου 4 kb μακριά από το γονίδιο Cdkn1b. Το γονίδιο Cdkn1b κωδικοποιεί για το p27, έναν καλά μελετημένο κυκλίνο – εξαρτώμενο αναστολέα κινάσης. Το p27 έχει μελετηθεί εκτενώς στο νευρικό σύστημα και έχει καθιερωθεί ως σημαντικός παράγοντας στην προώθηση της εξόδου από τον κυτταρικό κύκλο, στη νευρωνική διαφοροποίηση και στη μετανάστευση των νευρικών κυττάρων. Είναι πολύ ενδιαφέρον ότι τόσο το Lockd όσο και το p27 εκφράζονται διαφορικά κατά την ανάπτυξη του εγκεφάλου του μυός, αλλά τα προφίλ έκφρασής τους είναι ουσιαστικά αντίθετα. Λόγω της εγγύτητας των γενετικών τόπων του Lockd και του p27 και λόγω της συμμετοχής του p27 στη διαδικασία για την έξοδο από τον κυτταρικό κύκλο, αρχικά χρησιμοποιήσαμε N2A κύτταρα, μια ταχέως αναπτυσσόμενη κυτταρική σειρά νευροβλαστώματος μυός για να μελετήσουμε το Lockd και την πιθανή του σχέση με το p27.

Πράγματι, κατά την υπερέκφραση του lncRNA Lockd στην κυτταρική σειρά N2A, ο πολλαπλασιασμός αυξάνεται σημαντικά σε σχέση με τις συνθήκες ελέγχου. Επιπλέον, υπό τις ίδιες συνθήκες, η έκφραση του p27 καταστέλλεται σημαντικά, προτείνοντας ότι το Lockd καταστέλλει την έκφραση του p27, το οποίο με τη σειρά του οδηγεί σε αυξημένο πολλαπλασιασμό, καθώς το p27 προάγει φυσιολογικά την έξοδο από τον κυτταρικό κύκλο. Καθώς ο πολλαπλασιασμός είναι μια εξαιρετικά κρίσιμη διαδικασία για τα νευρικά βλαστικά κύτταρα κατά την ανάπτυξη του εγκεφάλου, εξετάσαμε επίσης την έκφραση του Lockd σε πρωτογενείς καλλιέργειες νευρικών βλαστικών κυττάρων.

Όντως, το Lockd εκφράζεται σε καλλιέργειες νευρικών βλαστικών κυττάρων, αλλά το πιο ενδιαφέρον στοιχείο είναι ότι η γονιδιακή του έκφραση μειώνεται σημαντικά σε συνθήκες καλλιέργειας χωρίς αυξητικούς παράγοντες σε σύγκριση με την καλλιέργεια παρουσία αυξητικών παραγόντων. Επιπλέον, κατά την υπερέκφραση του Lockd που πραγματοποιήσαμε σε νευρικά βλαστικά κύτταρα, ο πολλαπλασιασμός αυξάνεται και η έκφραση του p27 καταστέλλεται, όπως παρατηρήσαμε στα πειράματα με την κυτταρική σειρά N2A. Αυτά τα πρώτα ευρήματα αποκαλύπτουν μια συναρπαστική σχέση του Lockd με το p27, αλλά και ένα σημαντικό ρόλο αυτού του lncRNA στον πολλαπλασιασμό των N2A κυττάρων και των νευρικών βλαστικών κυττάρων.

Εν κατακλείδι, τα δεδομένα μας δείχνουν ότι τα lncRNAs είναι νέοι βασικοί παράγοντες στη διαφοροποίηση και τον πολλαπλασιασμό κατά τη διάρκεια της ανάπτυξης του εγκεφάλου και παρέχουμε τουλάχιστον δύο τέτοια παραδείγματα. Το Lacuna, ένα νέο lncRNA, είναι απαραίτητο για την έκφραση του μεταγραφικού παράγοντα Tbr2 / Eomes και αναστέλλει τη διαφοροποίηση των νευρικών βλαστικών κυττάρων και το Lockd, ένα ήδη μελετημένο lncRNA σε άλλο σύστημα, επηρεάζει αρνητικά την έκφραση του p27 και προάγει τον πολλαπλασιασμό. Η μελέτη μας παρέχει πληροφορίες για τη συμμετοχή των lncRNAs στην οργανογένεση του κεντρικού νευρικού συστήματος και δείχνει ότι τα lncRNAs μαζί με τα γονίδια που κωδικοποιούν για πρωτεΐνες σχηματίζουν ρυθμιστικά δίκτυα με σημαντικές λειτουργίες στα νευρικά βλαστικά κύτταρα και στην ανάπτυξη του εγκεφάλου.

Ευχαριστίες

Επέλεξα να γράψω τις ευχαριστίες στη μητρική μου γλώσσα, γιατί οι άνθρωποι που βοήθησαν στην ολοκλήρωση αυτής της διατριβής είναι αγαπημένοι φίλοι και συνεργάτες και θα ήθελα να τους ευχαριστήσω από καρδιάς. Η εξάχρονη περίοδος της διδακτορικής μου διατριβής ήταν η πιο δύσκολη και η πιο οδυνηρή περίοδος της ζωής μου, ενώ η ίδια διαδικασία του διδακτορικού ήταν το πιο ευχάριστο και παιχνιδιάρικο πράγμα που μου συνέβαινε. Ταυτόχρονα, όμως, ήταν μια περίοδος που με καθόρισε ως άνθρωπο και ως προσωπικότητα, που με σκληραγώγησε, μου χάρισε ένα σωρό όμορφες και ενδιαφέρουσες γνώσεις και εμπειρίες και μου γνώρισε αξιόλογους, γενναιόδωρους και θαυμάσιους ανθρώπους.

Πρώτα από όλα, θα ήθελα να ευχαριστήσω τον Πάνο, γνωστό και ως Δρ. Παναγιώτη Πολίτη, για όλους τους λόγους που ευχαριστεί ένας υποψήφιος διδάκτορας τον μέντορά του, αλλά κυρίως γιατί το εργαστήριό του είναι μια όμορφη και ζωηρή κυψέλη όπου δεν χωρούν ανταγωνισμοί και κακίες και γιατί αυτό που μετέφερε σε μένα, όπως και σε όλους τους άξιους συναδέλφους, είναι η ανιδιοτελής αγάπη για την επιστήμη, τον άνθρωπο και την φύση και η επιλογή να προχωράς στον επαγγελματικό στίβο με καλοσύνη και αξιοπρέπεια. Προσωπικά, μου στάθηκε ως εγκάρδιος φίλος σε όλα τα άσχημα και χάρηκε με τις επιτυχίες μου ειλικρινά. Τον ευχαριστώ, τον θαυμάζω και εύχομαι να μοιραστούμε πολλές ακόμα στιγμές στο μέλλον.

Θα ήθελα να ευχαριστήσω και τα υπόλοιπα μέλη της τριμελούς μου επιτροπής, τον επιβλέποντά μου κ. Γιώργο Διαλλινά και τον κ. Σπύρο Ευθυμιόπουλο. Και τους δύο έχω την τιμή να τους γνωρίζω από τα φοιτητικά μου χρόνια, ενώ ο κ. Διαλλινάς έχει συμβάλει τα μέγιστα σε όλη την ακαδημαϊκή μου πορεία και πάντα με στήριζε και με παρότρυνε. Η συμβολή και των δύο στην περάτωση της διατριβής ήταν σημαντική και τους ευχαριστώ από καρδιάς.

Θα ήθελα να ευχαριστήσω και τα υπόλοιπα μέλη της επταμελούς μου επιτροπής, την κα Παπαζαφείρη, την κα Θωμαΐδου, την κα Εμμανουηλίδου και τον κ. Σεραφειμίδη για την προθυμία τους να συμμετάσχουν, τα σχόλια και την καλοπροαίρετη κριτική τους. Ειδικά για τον Γιάννη Σεραφειμίδη, χαίρομαι πολύ για τη συμμετοχή του, αφού η συνεργασία μας ξεκίνησε χρόνια πριν κατά τη διάρκεια του Master μου και ακόμα τον θαυμάζω και τον συμβουλεύομαι, ειδικά στα πιο δύσκολα που είναι η ειδικότητά του.

Για το εργαστήριό μου, το Politis Lab «με το όνομα», όσα και να πω θα είναι λίγα. Θα ήθελα να ευχαριστήσω κάποια από τα παλαιότερα μέλη, τους ανθρώπους που με υποδέχθηκαν ως «καινούργια

του εργαστηρίου» και με καλωσόρισαν: Την Δάφνη Αντωνίου, την Μυρτώ Ρίζου, τον Θανάση Στεργιόπουλο, τον Πέτρο Μουστάρδα και τον κ. Χαρώνη, που μας λείπει πολύ και που μας έχει φροντίσει όλους. Επίσης, θα ήθελα να ευχαριστήσω την πιο όμορφη και γλυκιά τεχνικό του ΙΙΒΕΑΑ, την Βαλέρια Καλτεζιώτη για τη συνεργασία μας και την φιλία μας όλα αυτά τα χρόνια, τον Γιώργο Μπάρκα για τα αστεία μας και την καλή μας γειτονία, την γκρίνια και τα πειράγματα, την Ισμήνη Ροζάνη για την καλή διάθεση και τη βοήθειά της, την Τίνα Τσαμπούλα, για τις συζητήσεις -επιστημονικές και μη-, τα χειρουργεία μας, την καλή μας συνεργασία και αλληλοβοήθεια. Θα ήθελα να ευχαριστήσω και νεαρότερα μέλη του εργαστηρίου, που «πέρασαν» από το πρότζεκτ αυτό έστω για λίγο και άφησαν το επιστημονικό τους αποτύπωμα, αλλά κυρίως την καλή τους διάθεση: την Δώρα Μανωλάκου, τον Θωμά Γιώτη, την Βασιλίνα Φαράντζου, όπως και τον Ζακ Ταραμπούλους και την Εύη Κακούρη. Υπάρχουν και τρία άτομα στο εργαστήριο αυτό, χωρίς τα οποία η ζωή μου θα ήταν εντελώς διαφορετική και πιθανώς και το διδακτορικό ετούτο. Ο Δημήτρης Γκίκας, η Άρτεμις Μιχαήλ και η Ποπιάννα Τσιορτού. Δεν ξέρω πώς να ευχαριστήσω αυτούς τους ανθρώπους που κάποιες φορές με πήραν από το χεράκι, άλλες με πήραν αγκαλιά, ίσως κάποιες και σηκωτή. Οι άνθρωποι αυτοί είναι πλέον για μένα οικογένεια, τους ευχαριστώ πάνω από όλα για την αγάπη τους και εύχομαι να την ανταποδώσω. (Εντάξει, η Άρτεμις συμμετέχει στη δημοσίευση – κάτι θα πάρει και από εκεί.)

Εκτός εργαστηρίου, αλλά εντός ΙΙΒΕΑΑ, θα ήθελα να ευχαριστήσω τον φίλο μου Νικόλα (Νίκος Μαλισσόβας), τον Στέλιο Ραβανίδη, τον Φαίδωνα Κιτάντζη, την Ελένη Βασιλάκη, την Αλεξία Πολυσίδη, την Μαρίνα Πανταζοπούλου, τον Φοίβο Μπορμπόλη, τον Τάσο Δελλή, την κ. Λεβέντη και τη Βάσω, προφανώς τον Παύλο Αλεξάκο (στον Παύλο οφείλουμε όσοι δουλέψαμε με πειραματόζωα στο ΙΙΒΕΑΑ ένα άγαλμα) και το υπό ίδρυση σωματείο συμβασιούχων του ΙΙΒΕΑΑ. Θα ήθελα να ευχαριστήσω και παλαιότερους συνεργάτες, τη Βίβιαν Αναστασίου, τον Παναγιώτη Γιαννόγκωνα, τον Δρ. Αντώνη Γαβαλά και τους αγαπημένους Dr. Andrew MacCabe και Dr. Marga Orejas. Επίσης, θα ήθελα να ευχαριστήσω τον Δρ. Χατζή και το εργαστήριό του στο Φλέμινγκ για την προσφορά των πλασμιδίων, αλλά και τους φορείς που χρηματοδότησαν εμένα προσωπικά ή αυτό το πρότζεκτ (Fondation Sante, ΕΣΠΑ, ΙΚΥ) με την ελπίδα να είναι ευκολότερη και πιο λειτουργική η χρηματοδότηση των υποψήφιων διδακτόρων στο άμεσο μέλλον.

Επιπλέον, θα ήθελα να ευχαριστήσω κάποιους ανθρώπους εκτός του επιστημονικού χώρου. Για παράδειγμα, τον ψυχολόγο μου Ηλία (Ρήγο), την ψυχολόγο μου Μαρία (Σταυρουλάκη) και κάποια πολύ ξεχωριστά πρόσωπα, την Έλενα, την Ρία, την Ναταλί, το Μαράκι, την Άσπα για πολλές ενέργειες και αλληλεπιδράσεις που χρειάζεται μια ξεχωριστή διατριβή για να ειπωθούν. Επίσης, θέλω να ευχαριστήσω

τον αδερφικό μου φίλο και προσφάτως δικηγόρο μου, Χάρη Κολλιαστάση, που προσπάθησε να με ελαφρύνει από πολλά και διάφορα προκειμένου να γράψω τη διατριβή μου. Θέλω να ευχαριστήσω τις αγαπημένες μου φίλες, Στεφανία Πατέρα και Θεοδώρα Τζαναβάρη, συνάδελφοι και συνοδοιπόροι, αλλά κυρίως μεγάλες καρδιές και γρήγορα μυαλά που είναι πάντα δίπλα μου και με στηρίζουν σε όλα. Τις αγαπώ πολύ και τις δύο. Επίσης, θέλω να ευχαριστήσω την Κατερίνα Κισσανδράκη και τον Δημήτρη Χατζηχρήστο για τη φροντίδα τους, τον συνάνθρωπο Στέφανο Κουτρή για την κατανόησή του και τη σπάνια αυτή σύνδεση και την οικογένεια Παρασκευάδη (Στέφανος, Βάλια, Πάρις) για την αγάπη τους.

Πολλές ευχαριστίες οφείλω και στον σύντροφό μου, τον Κωστή Κουτρή, που δεν έλειψε στιγμή από κοντά μου και που ήθελε να ολοκληρωθεί η διατριβή αυτή περισσότερο και από μένα. Τον ευχαριστώ για πολλά, αλλά σε σχέση με την παρούσα διατριβή, κυρίως επειδή συνέχιζε να πιστεύει σε μένα όταν ήμουν έτοιμη να τα παρατήσω. Τέλος, ευχαριστώ τους δύο μου λατρεμένους γονείς, Γεωργία Ρηγάκη και Γιάννη Νίνο, για όλα όσα μου δίδαξαν και μου πρόσφεραν, για την αγάπη, την ελευθερία και τη στήριξή τους σε όλη μου την πορεία. Η διδακτορική μου διατριβή είναι αφιερωμένη στη μνήμη τους.

Αφιερωμένο σε όσους ανυπομονούσαν

να ξεφυλλίσουν το διδακτορικό μου

Και πιο πολύ σε εκείνους που δεν πρόλαβαν

Contents

Introduction

The mammalian brain is the most complex organ -or perhaps the most complex object- in the known universe. Its complexity could be depicted by the outrageous numbers of neurons and synapses that constitute the adult brain of humans and other mammalian species. Even if the human brain weights almost 2% of the total human body weight, this miraculous organ is capable of hundreds of functions and is the place where all human abilities, like language, art and science, were initially born.

These impressive outcomes and the basic functions that underlie beneath them are the result of functional neural circuits that mediate sensory and motor processing. These circuits are established via synaptic communication between neurons that have reached their regional positions and have extended axons and dendrites in order for them to communicate. These neurons have been generated during brain development and have migrated to self-organize and form the different brain structures together with the other cellular types of the central nervous system, astrocytes, microglia and oligodendrocytes. All these cell types are actually derived by neural stem cells, which have divided many times to drive brain growth and then, gradually, some of them stopped dividing and started to differentiate. Indeed, the differentiation of neural stem cells to neurons or glial cells is a major developmental process controlled by the interplay between extracellular signaling cues and intrinsic gene regulation circuitries.

Therefore, the scientific community is actively investigating in detail how these regulatory circuitries and networks are formed to control brain development and function. These networks are not only providing valuable information on how our brains are working, but also, they are involved in nervous system diseases, disorders and cancers. For a long time, it was thought that these networks are mainly based on cross-regulatory interactions between transcription factor genes that mediated neural stem cell expansion, neurogenesis and gliogenesis. However, novel insights and advances in sequencing technologies as well as data acquired by large-scale consortia on functional genomic elements studies have revolutionized the way we understand the activity, regulation and architecture of mammalian genomes. It has now become evident that the non-coding genome is not just “junk” as it was thought, but a part of this “uncharted” genome performs critical roles in the mammalian cells.

The notion that the genome employs its functions only via proteins and typical protein-coding genes appears progressively as a rather naïve oversimplification of a complex and mesmerizing system full of loops and networks that also involves RNA molecules. Indeed, our insight on activity, regulation and architecture of mammalian genomes has been completely revolutionized after latest advances in

sequencing technologies and data acquired by large-scale consortia on functional genomic elements studies like ENCODE and FANTOM [1-4]. Moreover, comparisons between transcriptomes and genomes of mammalian species have shown that approximately two-thirds of genomic DNA is transcribed, but only less than 2% is translated finally into proteins [5,6]. Additionally, even if alternative splicing and post-transcriptional regulation are taken into account, the level of organismal complexity among eukaryotes better correlates with the fraction of non-coding RNAs than with the sum of protein-coding genes [7].

In addition to the already known plethora and complexity achieved by protein-coding genes, their elements and interactions, a surprising number of non-coding RNAs (ncRNAs) has been identified. These ncRNAs are classified in small (scnRNAs) and long (lncRNAs) non-coding RNAs, which differ primarily in size, but also in function. Most of the sncRNAs function as post-transcriptional regulators in the cytoplasm [8], whereas lncRNAs act in a wide and diverse range of functions. Actually, lncRNA regulatory mechanisms have been found to extensively impact transcriptional, post-transcriptional and translational regulations of single genes and gene networks [9,10]. Therefore, lncRNAs provide cells with an extra tool to control the spatiotemporal regulation of genes, a critical requirement for neural stem cells during brain development.

Neural stem cells of the mammalian central nervous system

The mammalian central nervous system (CNS) is the most complex tissue of all living organisms. The CNS develops from a small number of cells that proliferate intensely before generating the main neural cellular sub-types. These multipotent cells, which also exist in the adult CNS, are known and considered as neural stem/progenitor cells (NSCs). NSCs first undergo symmetric proliferative divisions, resulting in the generation of two daughter stem cells, thus increasing the pool of stem cells. Later on, during embryonic development, these divisions are followed by a large number of asymmetric, self-renewing divisions generating a daughter neural stem cell and a more differentiated cell, such as a neuronal or glial progenitor. The induction of neural fate does not affect the proliferative capacity of cells. It is only later that committed precursors are instructed to become postmitotic, as progenitors exit the cell cycle, cease to proliferate and differentiate into neurons and glial cells. Both neural induction and initiation of differentiation pathways, either neurogenic or gliogenic, appear to be connected to cell cycle control systems that regulate whether NSCs will maintain their proliferative ability or differentiate into the appropriate neural cell type.

A large number of molecules, such as transcription factors, non-coding RNAs and signaling pathways participate during this differentiation procedure in order for the cells to maintain a neuronal, glial, or a stem cell fate. This regulatory network is essential for the generation of the appropriate number of neurons, neural subtypes and proper wiring of neuronal circuits in the CNS [11-14].

Differentiation of neural stem cells

Neural stem cells in the embryonic and early postnatal brain generate neurons and glia, including astrocytes and oligodendrocytes. To do so, NSCs must transit from a proliferative and multipotent state to fully differentiated neurons (neurogenesis) and glia (gliogenesis). During neurogenesis, neurons are produced from early embryonic development until early postnatal stages, with only a few neurogenic zones enduring in the adult [15]. On the contrary, gliogenesis starts later in embryogenesis and persists in postnatal stages, with low but extensive generation of astrocytes and oligodendrocytes also in the adult brain [16].

Early in the development of the mammalian embryo, the notochord induces neural fate of the above ectoderm. Then, the neural plate starts to form the distinctive regions of the CNS and undergoes neurulation to develop the neural tube. The neural tube constitutes a specific epithelium made up of neuroepithelial cells (NECs) that shift their nuclei according to the cell cycle phase. Before dividing, NECs move their nuclei to the ventricular surface in order to enter mitosis. In the beginning of neurogenesis, these cells turn into radial glial cells (RGCs) that generate all neurons and glial cells, directly or indirectly. More specifically, RGCs divide asymmetrically, generating a RGC daughter cell and a differentiating cell. The latter signifies a neuron or an intermediate progenitor (IP), which is a more fate-restricted progenitor. IPs divide generally symmetrically to give rise to two neurons and hence, to double the neuron output (Introductory scheme 1) [15,17].

Except of neurons, glia is a very important component of the CNS comprising at least 50% of the cells in the brain. Astrocytes are specified from RGC population in response to transcription factors gradient and signaling cascades in the cortical sub-ventricular zone. After specification, astrocyte precursors move away from the centers of germination and start to differentiate. This lineage commitment is manifested by the expression of GFAP marker and defined by the induction of a number of pro-astrogliogenic signaling pathways, such as JAK-STAT3 and/or BMPs, but also inhibition of other cell fates. Interestingly, both oligodendrocyte progenitors and astrocyte precursors are proliferating robustly

after their specification. Actually, astrocytes -unlike other brain cell types- are capable of mitosis even in adulthood. Additionally, astrocytes are crucial in repair processes of the CNS providing homeostasis in a pathological environment. Reactive astrocytes proliferate after injury and also display hypertrophy and tiling disorganization, but these processes also result in loss of normal functions [16,18,19].

Transcription Factors with crucial roles in neurogenesis: The Tbr2 paradigm

The specific patterning of the embryonic CNS through gradient of morphogens (e.g. Fgfs, Wnts, Shh, BMPs) leads to regional expression of transcription factors that instruct NPCs to generate specific cell types during neurogenesis [11]. One of the master regulators is Pax6 transcription factor, which is expressed in many regions of the CNS, like the forebrain, the hindbrain and the retina [20]. Pax6 promotes RGC proliferation, spindle orientation, but also neurogenesis by inducing the expression of proneural genes, such as Neurogenins [21]. But besides radial glia progenitor cells, the developing neocortex contains an additional type of progenitor cells for neurons, intermediate progenitor cells (IPs). This type of progenitor cells are derived by radial glia and generate only neurons – specifically, glutamatergic projection neurons (PN)- (in contrast to RGCs that generate neurons and glia) [22].

The transition from RGC to IP is linked to downregulation of Pax6 and upregulation of Tbr2/Eomes, a T-box transcription factor. Subsequently, the later transition from IP to postmitotic neuron is associated with downregulation of Tbr2 and upregulation of Tbr1 [22]. Actually, Tbr2 is transiently expressed during neurogenesis in the SVZ (subventricular zone), initiating between E10 and E12 in a small population of differentiated preplate neurons that includes Cajal-Retzius cells and decaying after E17 [22]. Tbr2 peak of expression in the CNS is detected between E12 and E16 in IPs, coinciding with the peak of cortical neurogenesis in mice.

Nevertheless, the role of Tbr2 in development is far greater than its contribution to cortical neurogenesis. In mice, Tbr2 is initially expressed in the trophectoderm at the blastocyst stage and, following implantation, it becomes restricted to the extra-embryonic ectoderm. Then, Tbr2 is induced within the proximal posterior epiblast. During gastrulation, Tbr2 is confined to the primitive streak and nascent mesoderm and later, it is limited to the anterior primitive streak before its abrupt downregulation. Its importance in trophectoderm lineage explains why Tbr2 loss-of-function mutants arrest at implantation stage [23]. Further studies on conditional Tbr2 loss-of-function mutants showed

that Tbr2 is also essential during anterior-posterior axis formation, epithelium-to-mesenchyme transition (EMT) and definitive endoderm specification [24].

Conditional inactivation of Tbr2 in the CNS revealed its roles during these very early or later developmental stages. CNS-specific Tbr2 inactivation does not affect viability nor fertility but causes microcephaly (result of lower proliferation in mutant SVZ) and behavioral abnormalities (e.g., aggressiveness, infanticide, hyperactivity). Moreover, neuronal output is reduced, neuronal differentiation is defective and dentate gyrus development is severely compromised [25]. More recent data showed that Tbr2 is necessary for the differentiation of projection neurons (PN), the specification of cell subtypes and the repression of IP-specific TFs, including its upstream Pax6, while it regulates hundreds of genes including PN-specific genes [26].

In humans, Tbr2 is detected in embryonic brain, specifically in the forebrain floorplate, at the 7th week of development. Later, at 12.5 weeks, it is observed in the mantle layer and migrating neuroblasts of the telencephalon. As expected, there are differences between Tbr2 expression in mice and humans, but still human Tbr2 seems to be important for late neuronal development. Of note, there is a homozygous chromosomal translocation that disrupts proper Tbr2 expression in humans and causes severe neurodevelopmental defects (microcephaly, polymicrogyria, corpus callosum agenesis, cognitive deficits, hypotonia) [27].

It is also worth noting that Tbr2 is encountered in other tissues as well. Besides its contribution in definitive endoderm specification during gastrulation, Tbr2 also marks the cardiac mesoderm and facilitates the generation of cardiovascular progenitors [28]. Moreover, Tbr2 is found to be involved in processes of the immune response. Indeed, it is expressed by resting and activated Natural Killer (NK) cells as well as activated CD8+ T cells [29] and it is reported to promote the development of central memory cells [30]. Additionally, it regulates a specific CD8+ T cell population in the thymus [31] and it is necessary for the generation of invariant Natural Killer T (NKT) cells and their differentiation in peripheral tissues [32].

Coupling neurogenesis and cell cycle exit

Neurogenesis, meaning the generation of new neurons in the embryonic brain cortex, requires that progenitor cells will exit the cell cycle and initiate the appropriate programs of differentiation and migration. There are multifaceted interactions between cell cycle components and regulatory factors of

neural development. Most importantly, cyclin-dependent kinase inhibitors (CKIs) are the major controllers of cell cycle progression and are divided into Cip/Kip and INK4 families [33].

$p27^{Kip1}$ is the most essential CKI in cortex development. Its expression in NPCs in the cortex define two parameters; the cell cycle length and the probability of cell cycle exit, therefore the birth of cortical projection neurons [34]. Additionally, except of its well-established role in NSC proliferation, $p27^{Kip1}$ also affects cell fate choices and differentiation, but also neuronal migration in brain development [35,36]. Indeed, $p27^{Kip1}$ functions as a modular protein that regulates and couples differentiation and migration pathways of cortical projection neurons [37] and it is reported even to stabilize NGN2 protein in cortical progenitors with subsequent results in neuronal differentiation [38].

Biogenesis and function of lncRNAs

LncRNAs are an heterogeneous class of RNA transcripts of at least 200 nucleotides in length (sometimes they exceed the 100 kilobases) that lack an evident open reading frame (ORF). Just like the typical protein coding genes, they undergo 3' polyadenylation, 5' capping, splicing modifications and their function is dependent on their transport to the cytoplasm [39]. Their transcription is conducted by RNA polymerase II and their location varies among many genomic regions: introns of protein coding genes, anti-sense to other genes, intergenic regions [40-42], promoters [43], enhancers [42], gene regulatory regions like UTRs [44], even chromosomal regions like telomeres [45]. Except for the genomic locus, lncRNAs are classified according to their mechanism of function. LncRNAs have been found to act through a wide range of mechanisms, such as transcriptional regulation, alternative splicing, nuclear-cytoplasmic shuttling, mRNA degradation, RNA decoys, translational inhibition and regulation of protein activity [46,47]. As these weren't enough, lncRNAs also act as precursors for miRNAs and snoRNAs [9] (Introductory scheme 2). Hence, it is obvious that lncRNAs present a spectacular functional diversity, possibly due to their folding capabilities resulting in diverse three-dimensional structures and their modular organization. These features, together with their intrinsic nucleic acid nature, allow them to perform molecular interactions with proteins and other nucleic acids. And as they exceed the 200-nucleotides limit, their length is enough to include multiple functional domains, so that one lncRNA can coordinate the spatiotemporal activity of many factors by -simultaneous or not- interaction with different molecules.

In terms of transcription regulation, lncRNAs can act both *in cis* and *trans*, influencing the expression of local or distal genes respectively, or of whole genomic regions like during XIST-induced X-chromosome inactivation [48]. LncRNAs not only recruit or expel effectively DNA methyltransferases, chromatin modification complexes and transcription factors, but also they act as scaffolds bringing together these various factors, finally resulting in selective activation or repression of genes [49]. More specifically, some lncRNAs have been found to facilitate the transcription of genes by recruiting histone H3K4 methyltransferases [50,51]. On the contrary, there are lncRNAs, like the well-studied HOTAIR, that silence *in cis* or *trans* protein coding genes by interacting with the PRC2 complex [52]. Besides histone modification complexes, lncRNAs also interact with transcription factors, DNA methyltransferases and other DNA/RNA binding proteins, facilitating or preventing their binding to the DNA and targeting DNA methylation [49,53]. There is such an example in the central nervous system, the lncRNA Dali, which is reported to promote neuronal differentiation and controls *in trans* the DNA methylation of CpG island-associated promoters by interacting with DNMT1 DNA methyltransferase [54].

As stated above, lncRNAs can act also through transcriptional and post-transcriptional control including alternative splicing, mRNA stability, nuclear-cytoplasmic shuttling and translational control (Introductory scheme 2) [55-57]. There are many reported lncRNAs expressed in the brain that are localized in the nucleus and they are related to splicing regulation (NEAT1, MALAT1, GOMAFU, SAF) [9,56,58]. The mechanisms are mainly three: modulating splicing factors influencing their activity, regulating the interactions of such factors with other splicing factors and pre-mRNAs as well as chromatin remodeling factors [56]. However, it is known that lncRNAs act also in the cytoplasm and that they can target mRNAs affecting their stability negatively or positively [59]. For example, ½-sbsRNAs and GADD7 reduce the stability of mRNAs [60,61], while BACE1-AS and TINCR promote this feature [62,63].

At the translational level, lncRNAs are important players as well, although the putative mechanisms are still less well defined. Especially for neurodevelopment and neuronal function, translational regulation is crucial in terms of spatiotemporal management of protein dynamics. Some interesting paradigms include the antisense lncRNA AS-Uchl1, which targets Uchl1 mRNA and promotes cap-independent translation [64] and lincRNA-p21, which causes ribosome drop-off on its targets [65]. Other mechanisms involve competition for miRNA binding [66] and being the precursor for small ncRNAs and miRNAs [67] (e.g. the famous H19 [68]).

LncRNAs in brain and CNS development

The central nervous system (CNS) is characterized by a huge variety of neuronal and glial cell subtypes, but also it is the most diversified and complex organ in terms of ncRNAs [9]. Interestingly, lncRNA expression in tissues is more specific than the mRNA expression and also, lncRNAs show specific temporal expression patterns during brain development [39]. Intriguingly, the lncRNAs that are expressed in the brain are preferentially harbored by genomic loci in vicinity of brain-specific, transcriptionally active during development, protein coding genes [69]. Generally, lncRNAs are reported to participate in a very broad spectrum of developmental processes of the brain (Introductory scheme 3).

Cis- and *Trans*-lncRNA regulatory networks in neuronal differentiation

Neuronal fate decision is finely controlled in space and time so that the progenitor cells choose between self-renewal and differentiation. Embryonic stem cells are greatly used to study this process, the exit from pluripotency and the entrance to neural differentiation pathways. Interestingly, lncRNAs regulate the activation of gene regulatory programs that guide the progenitor cells sequentially and coordinately from the pluripotent state throughout the terminal cell types that constitute the mature mammalian brain. There is a long list of lncRNAs that are reported as necessary for establishing pluripotency or neural lineage specification [70-72]. It is widely accepted that pluripotency transcription factors like OCT4, SOX2 and NANOG are absolutely required in this process, but in turn lncRNAs appear to be able to contribute to these phenomena by regulating such transcription factors and/or chromatin modifiers in order to affect lineage-specifying genes *in cis* or *trans*.

There are some emblematic examples on lncRNA involvement in neuronal fate decision. First, the lncRNA Evf2, one of the first nervous system-specific lncRNAs that was studied *in vivo*, is reported to control the expression of Dlx5, Dlx6 and Gad1 (interneuron-specific genes) by *cis* and *trans* scaffolding mechanisms, through which MeCP2 (a methyl-CpG-binding protein) and DLX transcription factor are recruited to the appropriate regions. This is the reason why Evf2 disruption causes defective GABAergic interneuron specification and consequently, disturbs the equilibrium between excitatory and inhibitory neurons in the dentate gyrus and hippocampus [73]. Second, the lncRNA Pnky, which is expressed in the developing mouse and human brain and specifically in the nucleus of NSCs, is reported to control the balance between proliferation and neuronal differentiation in these cells through interaction with splicing regulator PTBP1 and therefore, alternative splicing [74]. Another in vivo study shows that linc-RNA Brn1b

controls the differentiation of delaminating neural progenitor cells *in cis*, as it regulates the expression of the neighbouring BRN1, a protein-coding gene related to cortical progenitor turnover [75].

Besides these fascinating *in vivo* studies, other groups have shed light on the link between lncRNAs and pluripotency transcription factors. Such an example is the lncRNA Rmst, which under its induction by REST, recruits SOX2 *in trans* targets like Dlx1, Ascl1, Hey2 and Sps, resulting in neural differentiation events [72]. Similarly, in ESCs, Tuna is reported to form a complex with NCL, PTBP1 and hnRNP-K RNA-binding proteins, which targets (*in trans*) and promotes Nanog, Sox2 and Fgf4 expression [76].

One of the most studied lncRNAs in the nervous system is Malat1, which was first reported as a highly expressed lncRNA in different types of neurons in mice. Malat1 is localized in nuclear speckles, a class of nuclear body located in pre-mRNA splicing factor-enriched regions of the nucleoplasm. A loss-of-function genetic model showed that Malat1 is not critical for development, as these mice showed only minor effects on Malat1-neighbouring genes. However, in mouse hippocampal neuron cultures, Malat1 knockdown resulted in reduced synapse density and dendrite growth. In a different system (in human lung tumor cells), MALAT1 was found to interact with components of PRC1, suggesting a possible mechanism of action [77-79].

Respectively early in the ncRNA biology field, lncRNAs were associated with neurogenesis. Actually, large screenings identified human lncRNAs that, upon knockdown, blocked the differentiation of embryonic stem cells (ESCs) into mature neurons [72]. In terms of mechanistic events, lncRNA-N1 and lncRNA-N3 bind to SUZ12 and REST, suggesting a model by which these lncRNAs recruit PRC2 to specific glial lineage genes and thereby, promote neurogenesis. On the contrary, lncRNA-N2 functions as a precursor for let-7, a proliferation arresting miRNA, and miR-125b, a neuronal differentiation promoting miRNA [80,81].

Apart from neurogenesis, lncRNAs are involved in oligodendrocyte specification as well. In one impressive study, Nkx2.2as (antisense to Nkx2.2) was reported to positively regulate oligodendrocyte specification and its overexpression resulted in increased populations of Nestin+ stem cells and a preference towards oligodendrocyte lineage during differentiation [82]. These are only some examples for the crucial role of lncRNAs in cell fate decision and stem cell turnover in neurodevelopment (Introductory scheme 4), conducted through a wide range of actions and mechanisms.

Ancient roles of lncRNAs in neurodevelopment

LncRNAs are present outside the class of mammals as well. For example, in zebrafish, genomic analyses reveal not only a large number of lncRNAs [83], but also sex-based differences in the expression of lncRNAs in mature zebrafish brain [84]. Outside the kingdom, in plants, there is a growing interest for lncRNAs with a focus on *Arabidopsis thaliana* and the involvement of lncRNAs in flowering time regulation [85], in cold exposure [86], in development and stress responses [87]. Even in yeast, lncRNAs are expressed and usually they affect *in cis* the transcription of protein-coding genes [88] and recently this field is expanding to filamentous fungi [89].

There is much debate on the human-specific lncRNAs and the highly accelerated regions of human genome (discussed later), but there is also valuable information on the highly conserved brain-expressed lncRNAs that show analogous spatiotemporal expression profiles from birds to mammals, suggesting ancient roles in brain development for these lncRNAs. Often, such lncRNAs originate from ultraconserved regions (UCRs) in the genome and regulate their nearby genes, which encode key developmental regulatory proteins [90,91]. A characteristic example is the above mentioned lncRNA Dlx6os1 (or Evf2), which is antisense to Dlx6 and downstream of Dlx5 in a mouse UCR. The Dlx genes (found also in *Drosophila melanogaster*) encode transcription factors with important roles in forebrain and craniofacial development. Dlx6os1 actually controls the expression of Dlx6, Dlx5 and Gad1 (glutamate decarboxylase 1 gene – responsible for GABA synthesis), acting both *in cis* and *in trans* [91]. The *in cis* action refers to the negative regulation of Dlx6, as hypothesized by their locus. The *in trans* action involves the recruitment by Dlx6os1 of DLX2 (an activator) and MECP2 (a repressor) on the locus in order to control Dlx5 and Gad1 expression. The crucial role of Dlx6os1 in neuronal activity was also shown *in vivo*, where loss of its function in mice resulted in defective synaptic inhibition and reduced numbers of GABAergic interneurons in postnatal hippocampus [73,91].

Taken together, the multidimensional functions of the lncRNAs meet the complex regulatory demands of the CNS and extensive study may reveal critical details of even more complex brain functions or of pathogenesis of neurodevelopmental and neurodegenerative disorders.

lncRNAs and disease

Most diseases and disorders have genetic backgrounds but are rarely attributed to a single gene or chromosomal abnormality. Usually, a combination of genetics and environmental factors defines the

risk of disease for each individual. A successful approach to this interaction is the field of epigenetics, where ncRNAs, and especially lncRNAs, are the new, promising key players. However, there are disorders, syndromes and diseases of all tissues and organs attributed to specific lncRNA mutations. For example, cartilage-hair hypoplasia is a recessively inherited disorder associated with bone and cartilage abnormalities and it is caused by mutations, insertions and duplications that result in reduced expression of RMRP, a lncRNA involved in rRNA maturation [92].

LncRNAs in brain cancers

As the functions of lncRNAs are so diverse that they include roles in regulatory mechanisms of cell growth, apoptosis, viability, inflammation and oxidative stress, they are also studied in relation to tumorigenesis. Especially due to their abundance in the CNS, lncRNAs are extensively studied in relation to nervous system cancers and malignancies, where they can also serve as biomarkers of prognosis and diagnosis, but also as therapeutic targets (reviewed in [93]).

For example, one of the most well-studied lncRNAs, HOTAIR, is found misregulated in many cancers - including the most aggressive cancer of the nervous system, glioblastoma multiforme. It is involved in histone modifications and chromatin remodeling that result in gene silencing and subsequent promotion of cancer cell proliferation, progression and metastasis [94,95]. Another example is H19, a lncRNA that is found upregulated in glioblastoma stem cells, where it promotes their stemness and their tumorigenic capacity [96]. Of course, we should also mention MALAT1, which is extensively studied in glioma as a tumor-suppressive lncRNA [97]. MALAT1 functions probably via suppression of ERK/MAPK pathway, a signal transduction pathway that regulates proliferation and invasion of cancer cells [98].

Besides these examples, cancer biology has recognized the importance of lncRNAs in brain (and other tissues) malignancies, especially their interaction with epigenetic modulators, their involvement in post-transcriptional regulation and their cross-talking with miRNAs. Undeniably, these studies have already suggested some potential therapeutic targets (e.g., MALAT1 and HOTAIR), but also particularly useful and reliable biomarkers that can even allow noninvasive diagnosis, which is of great interest in brain cancers.

LncRNAs in neurodegenerative/ neurodevelopmental diseases and disorders

Chronic neurodegenerative diseases affect millions of people and still, are considered incurable. The pathogenesis of these diseases involves genetic background and environmental risks. Biomedical research is studying lncRNAs in this field as well, not only as potential players in neurodegenerative processes, but also as putative therapeutic targets.

For instance, BACE1-AS is a well-known lncRNA due to its association with Alzheimer's disease. BACE1-AS is transcribed antisense to BACE1, a gene encoding for a protease that cleaves amyloid precursor protein (APP) and abnormal cleavage is associated with Alzheimer's disease. Many findings support the hypothesis that BACE1-AS increases the stability of BACE1 mRNA, resulting in increased protein production. Increased BACE1 levels lead to increased pathogenic APP cleavage products – amyloid β 1-42 - that (with a positive feedback mechanism) induce BACE1-AS expression, and in turn, BACE1 levels, which thereby further produce more pathogenic amyloids. Furthermore, BACE1-AS expression in the brain is 2-fold higher in patients with Alzheimer's compared to control [62]. Additionally, in Huntington's disease, some lncRNAs were identified to be differentially expressed as compared to control (e.g. TUG1, NEAT1, MEG3) and possibly their role is PRC2-mediated [99].

LncRNAs have also been associated with neuropsychiatric disorders like autism spectrum disorders, schizophrenia, intellectual disability, Rett syndrome, depression and anxiety disorder (reviewed in [100]). For example, in families with X-linked intellectual disability and autism spectrum disorders, there have been observed variants at the PTCHD1 locus on X-chromosome. On the antisense strand of this gene, there are 3 overlapping lncRNAs (PTCHD1AS1-3), which may regulate PTCHD1, a gene involved in synaptic and neuronal excitation, cognitive function and motor ability [101].

Another paradigm is Gomafu, a lncRNA with important role in brain development and neuronal function, which acts in the splicing of mRNAs as stated above. Gomafu is located in a chromosomal region associated with schizophrenia (22q12.1). It is known that this lncRNA binds directly to splicing factors and also, its dysregulation results in upregulation of DISC1 and ERBB4 schizophrenia-associated genes. Importantly, Gomafu was found downregulated in the cortex of schizophrenic patients (post-mortem), suggesting a role in the pathogenesis of this disorder. [102]

Of interest, some lncRNAs have also been associated with substance dependence disorders. For example, MALAT1 is upregulated in the brain of alcohol abusers [103]; NEAT1, NEAT2 and MEG3 are

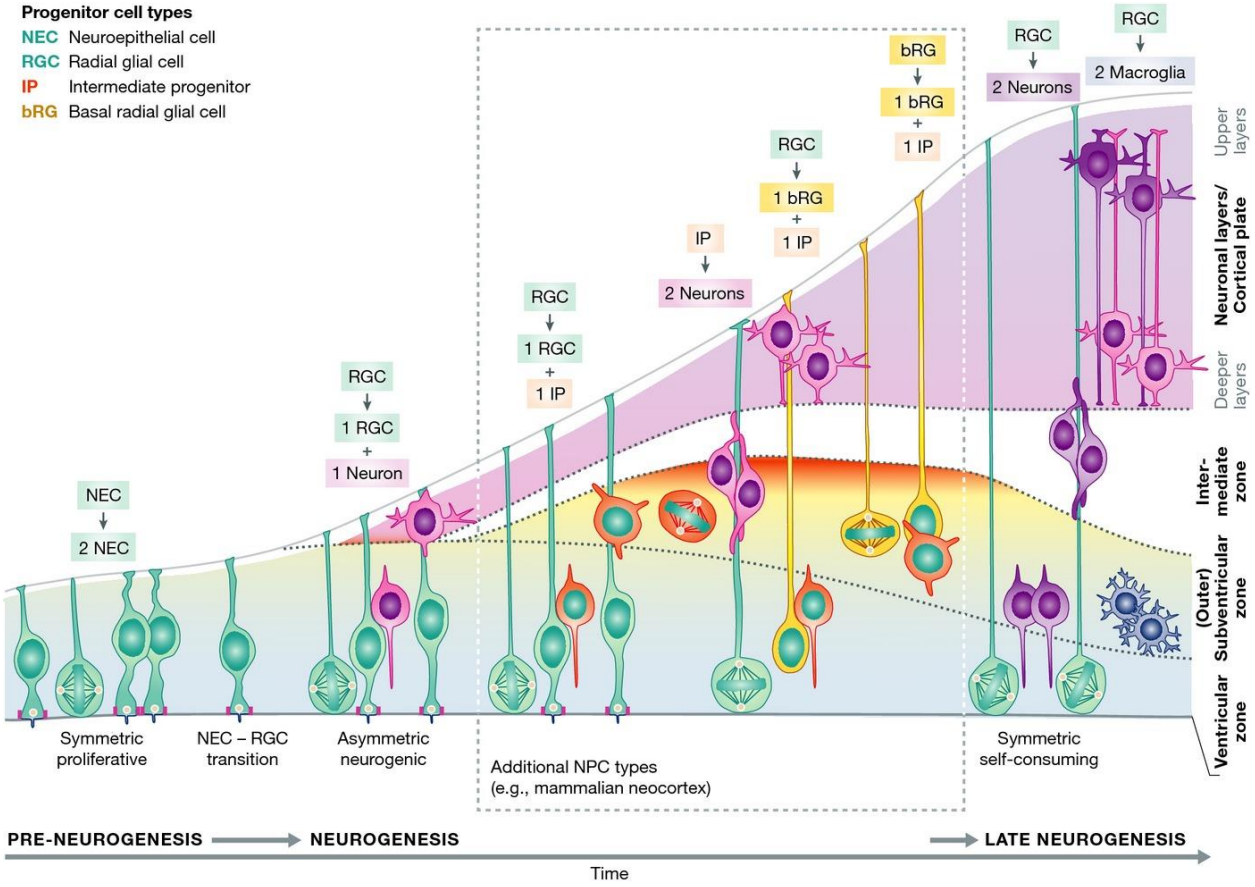
upregulated in the brain of heroin-dependent persons and NEAT2, MIAT, MEG3 and EMX2OS are upregulated in the brain of cocaine-dependent subjects [104].

LncRNAs implications in human brain evolution

LncRNAs display generally relatively low evolutionary conservation, although their splice-junction motifs [105] and their promoters -with the corresponding tissue specific binding sites- are highly conserved [70,106]. The low conservation, though, does not mean automatically lack of biological function. The broad spectrum of actions and functions of lncRNAs and their involvement in development and disease, together with their low evolutionary conservation suggest lncRNAs as important means of human brain evolution [107]. Most protein-coding genes expressed in the nervous system are highly conserved not only in primates, but across mammals [108-110]. In contrast, one third of human lncRNAs are present only in primates [106] including hundreds of human-only lncRNAs [111]. Furthermore, there are some lncRNA loci that have been positively selected during human evolution and some even positively selected within discrete populations [109,112]. A known example is HARF1, a positively selected lncRNA expressed in Cajal Retzius neurons during human embryonic cortical development that is proposed to be a human-specific cortical development driver [113]. On the contrary, many studies tried to find positive selected protein-coding genes significant to the nervous system in humans relative to primates and rodents, but it turned out that the lack of such genes was a much more interesting finding [107,114].

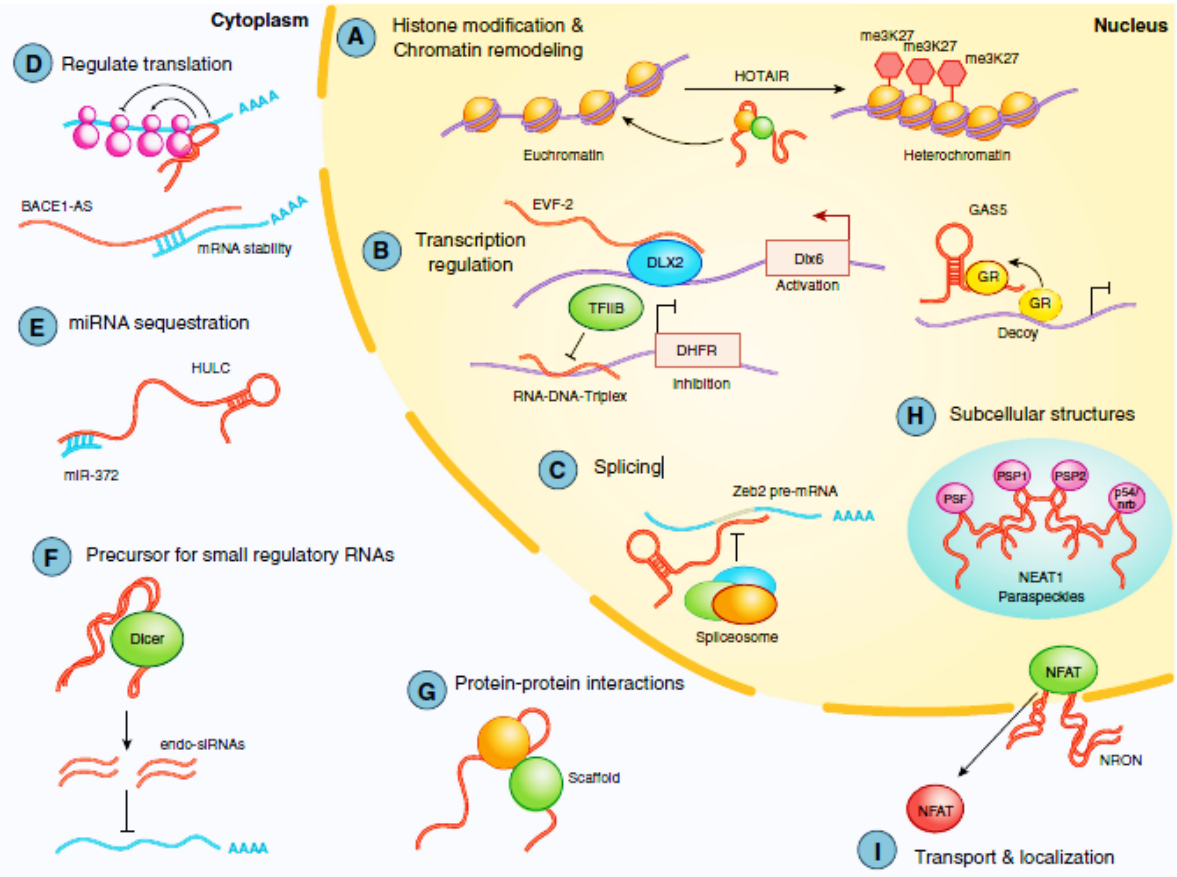
Introductory schemes

Introductory scheme 1



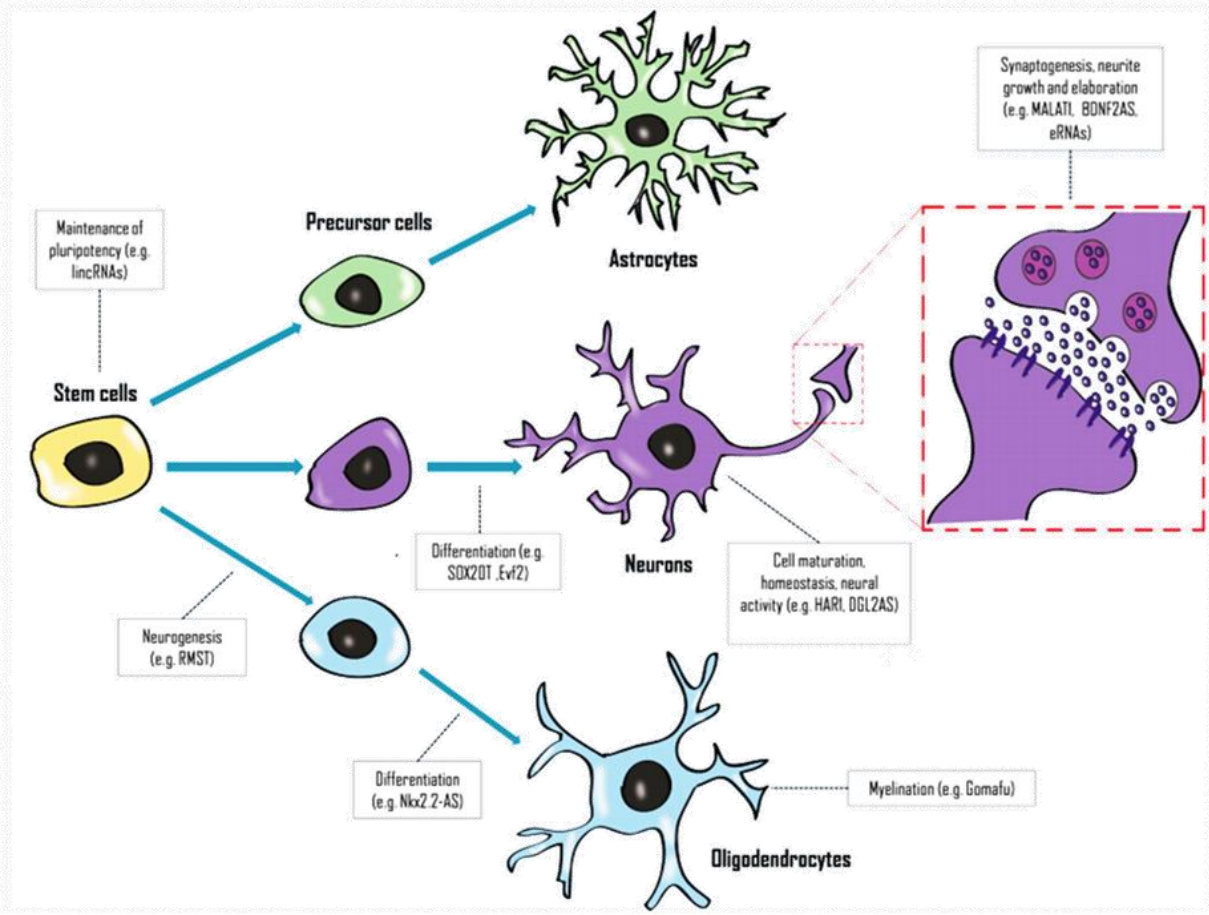
Introductory scheme 1: Overview of neurogenesis in the embryonic CNS (scheme from [15]).

Introductory scheme 2



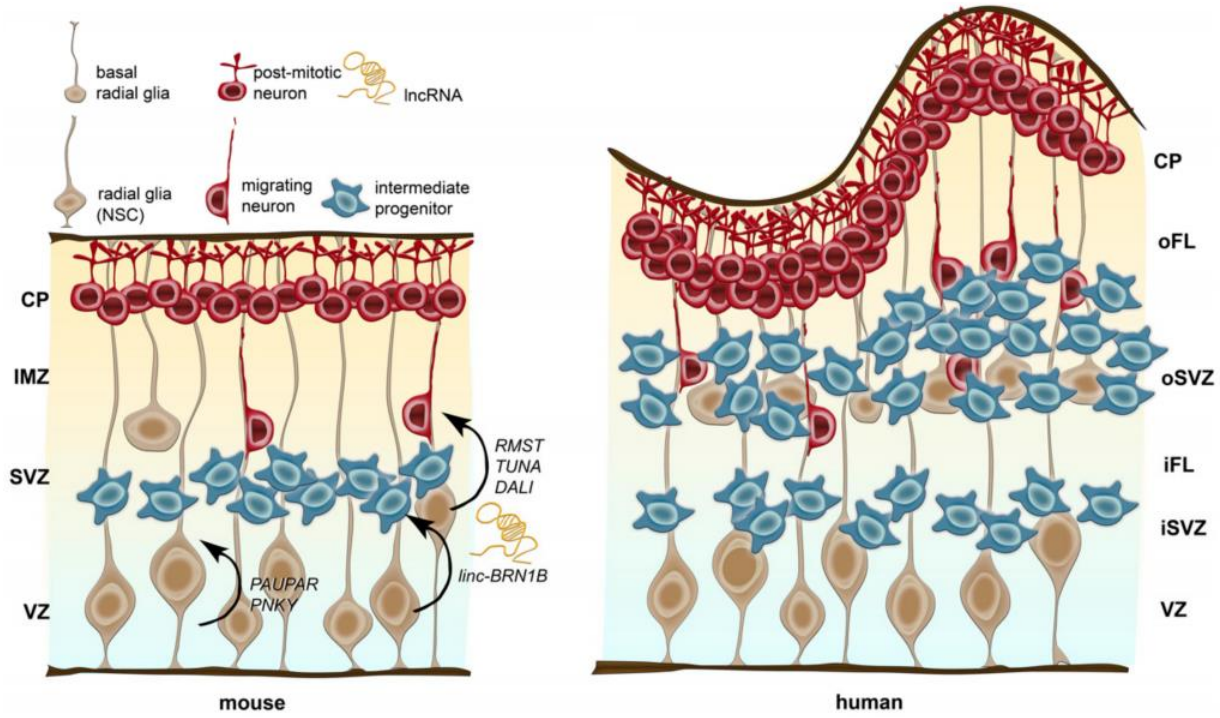
Introductory scheme 2: LncRNA cellular functions. (A) LncRNAs can guide chromatin remodeling complexes (e.g. HOTAIR). (B) lncRNAs can inhibit or promote the transcription of protein coding genes by recruitment or decoying of transcription factors, cofactors and RNA pol II (e.g. EVF-2, GAS5). (C) LncRNAs are involved in alternative splicing. (D) LncRNAs can pair with mRNAs affecting their stability and translation. (E) They can bind to miRNAs preventing their action. (F) They can form small, single or double stranded RNAs that behave as siRNAs. (G) They act as scaffolds for protein-protein interactions. (H, I) They act as scaffolds in subcellular structures (e.g. NEAT1) and transport of proteins (e.g. NRON). (Scheme from [47], adapted from [115])

Introductory scheme 3



Introductory scheme 3: LncRNAs are involved in the maintenance of pluripotency, neurogenesis, cell fate decision processes, maturation and differentiation. They participate in homeostasis and activity of neural cells. They also contribute to synaptogenesis, neurite growth, synaptic plasticity and signaling (Scheme from [100]).

Introductory scheme 4



Introductory scheme 4: Neurogenesis in mouse and human cerebral cortex and potential involvement of lncRNAs examples. The ventricular zone (VZ) includes radial glia cells, so called neural stem cells (NSCs) that generate neurons, intermediate progenitors and basal radial glia cells in mice and humans. The subventricular zone (SVZ), which includes the intermediate progenitors and the basal radial glia, presents differences between the two species. In humans, it is expanded and separated by the inner fibre layer (iFL) into an inner (iSVZ) and outer (oSVZ) SVZ. In rodents, postmitotic neurons migrate along the basal processes of the radial glia of the VZ and SVZ through the intermediate zone (IMZ) into the cortical plate (CP). In humans, postmitotic neurons have to pass some extra zones (iSVZ, iFL, oSVZ and oFL) to find CP. Rmst, Tuna, Dali are lncRNAs proposed to drive neuronal differentiation, whereas Paupar and Pnky are reported to control the balance between proliferation and differentiation of neuronal progenitor cells. Linc-Brn1b is involved in basal cortical progenitor turnover regulation and more specifically, in the differentiation of delaminating neural progenitor cells (figure from [107]).

Aim of the thesis

The principal aim of this PhD thesis is to better understand the involvement of long non-coding RNAs in mammalian brain development and especially in the gene regulatory networks that define the identity of neural stem cells.

The mammalian brain is the most complex organ of all living organisms. In recent years, a major effort has been made to elucidate the genetic regulatory networks that control this striking cellular complexity. For a long time, the scientific community believed that these networks are regulated solely by cross-interactions between a plethora of transcription factors. However, the evolution of new genome sequencing technologies and the access to experimental data through databases, such as ENCODE and FANTOM, have radically changed the way the scientific community deals with the organization, activity and regulation of the mammalian genome. It has now become clear that most of the genome is transcribed and produces a large number of regulatory RNA molecules that were not previously known. Among them, long non-coding RNAs appear to be involved in regulatory networks that control embryonic stem cell pluripotency, carcinogenesis, growth, and the function of many tissues and organs.

Although thousands of lncRNAs are expressed in embryonic and adult mammalian brain in a highly patterned and specific manner, they remain poorly characterized and their roles in brain development have not yet been studied. Also, many studies indicate that lncRNAs are involved in the pathophysiology of brain related diseases/disorders, and have played critical role in the evolution of mammalian brain. To further explore the links between lncRNAs, brain development and brain related diseases, we wanted to investigate the involvement of these molecules in the development of mouse brain. Therefore, here our main goal is to address how long non-coding RNAs affect the formation of mammalian brain during development.

In particular, based on initial observations in the literature which show that a significant proportion of lncRNAs have the ability to regulate neighboring genes *in cis*, we hypothesized that lncRNAs may be involved in the gene regulatory networks of neural stem cells (NSCs) through their ability to control the expression of genes encoding for transcription factors. Thus, we performed a series of RNAseq assays to detect lncRNAs that are expressed in the developing mouse brain and are transcribed from genetic loci adjacent to genes encoding for well-studied transcription factors involved in these networks. In this PhD

thesis, we aim at the systematic study of the role of these lncRNAs in gene regulation mechanisms in brain development in mammals.

To this end, the specific objectives of this thesis include:

a) Identification of lncRNAs expressed in neural cells and characterization of the changes in the expression profile of these lncRNAs during development of mouse brain (telencephalon).

b) Assessment whether the lncRNAs identified in the previous aim are required for brain development. Here we plan to examine in a comprehensive fashion the role of short-listed lncRNAs from previous aim in controlling the properties of mouse NSCs. We have previously developed experimental systems to isolate, culture and differentiate NSCs from the embryonic CNS of mice, and used these systems to study the molecular mechanisms that control proliferation, survival, cell-fate specification and differentiation of NSCs. This aim includes two specific objectives:

1: Overexpression of selected lncRNAs and analysis of their effects on *ex vivo* cultured NSCs.

2: Knockdown of selected lncRNAs using a CRISPR-dCas9-KRAB effector system and analysis of their effects on *ex vivo* cultured NSCs.

The rationale of this project is to provide insights into the involvement of lncRNAs in organogenesis and understand how lncRNAs and protein-coding genes form regulatory networks with important functions in neural cells. Understanding the role of lncRNAs in brain organogenesis could revolutionize the basic principles of developmental/cell biology and neuroscience.

Materials and Methods

Ethics statement

The study protocol took place in the animal facilities of the Center for Experimental Surgery of the Biomedical Research Foundation of the Academy of Athens. All animals were handled in strict accordance with good animal practice as defined by the relevant European and Greek animal welfare bodies.

Culture of NSCs, overexpression and knockdown studies

Neurosphere cultures from E14.5 mouse cortical tissue were prepared as described previously [116-118]. Proliferation or differentiation assays were performed after dissociation of NSCs to single cells, plating onto poly-L-lysine (Sigma) coated coverslips in 24-well plates at a density of 10^5 and further *ex vivo* culture for 2 or 3 days with or without Growth Factors, respectively, in a 37° C humidified incubator with 5% CO2. The cells were maintained in suspension in full medium with growth factors as follows: 1:1 mixture of Dulbecco's modified Eagle's medium (1 g/liter d-glucose, l-glutamine, pyruvate; Sigma), F-12 nutrient mixture (Sigma) plus 20 ng/ml human epidermal growth factor (EGF; R&D Systems) and 20 ng/ml human basic fibroblast growth factor (R&D Systems), 20 μg/ml insulin (Sigma), 1× B27 supplement (Gibco), 0.25 mM l-glutamine, and 1% penicillin/streptomycin to promote the production of the neurospheres. The neurospheres were passaged 2–3 times before the assays. Differentiated neurosphere cultures were maintained in minus growth factors conditions, the same as the full medium plus growth factors without human EGF and basic FGF, in order to promote differentiation.

For overexpression studies, the Lacuna lncRNA sequence and the Lockd lncRNA sequence were ordered to be cloned into pcDNA (GenScript) and then they were cloned into pCAGGs vector. Together with pCAGGs-Lacuna or pCAGGs-Lockd, a pCAGGs-GFP plasmid was used (3:1) in order to visually mark the transfected cells. Empty pCAGGs together with pCAGGs-GFP (3:1) were used as a control for the overexpression experiments.

For knockdown studies, a CRISPR-dCas9-KRAB effector system (kindly provided by Dr. Hatzis) was used. This system consists of two plasmids (1:1): a pHR-KRAB-dCas9-mCherry and a pU6-sgRNA-EF1Alpha-puro-T2A-BFP (without gRNA for control and with gRNAs against Lacuna sequence for Lacuna knockdown). gRNAs were designed using the GenCRISPR gRNA Design Tool to target the first exon of Lacuna sequence.

NSCs were transfected using an AMAXA electroporator (Lonza) with 6μg of total plasmid DNA per electroporation, according to manufacturer's instructions [116,117]. After electroporation, they were

incubated overnight in full medium with 1% FBS in order for them to surpass electroporation shock and then they were incubated according to the experiment.

N2A cell line cultures

N2A cells were cultured in DMEM (Low Glucose, BIOSERA) medium supplemented with 10% (vol/vol) heat-inactivated FBS (Biosera) and pen-strep (100 U /ml and 100 μg /ml, respectively; Invitrogen). All cell lines were transfected with Lipofectamine 2000 (11668027) according to the manufacturer's protocol. For overexpression studies in N2A cells, the sequences of the selected lncRNAs were cloned into pCAGGs vector. Together with pCAGGs-LncRNA, a pCAGGs-GFP plasmid was used (3:1) in order to visually mark the transfected cells. Empty pCAGGs together with pCAGGs-GFP (3:1) were used as a control for the overexpression experiments.

RNA extraction and real-time RT-qPCR analysis

Total RNA was isolated by cells and tissues with TRI reagent solution (AM9738, Ambion/RNA, Life Technologies) according to manufacturer's instructions followed by treatment with RQ1 DNase (Promega, Madison, WI, USA). RNA concentration and purity was measured by Nanodrop 2000c (Thermo), and 1.5 μg was used for cDNA synthesis using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, USA) together with random hexamer primers. Quantitative Real time RT-PCR analysis was performed in a LightCycler 96 Instrument (Roche). Measured values were normalized using beta actin or Gapdh and RPL13A mRNA levels as internal references.

Primers that were used for real-time RT-qPCR are presented in the following table:

Genes	**Sequence**	
beta actin	Forward	CCCAGGCATTGCTGACAG
	Reverse	TGGAAGGTGGACAGTGAGGC
Gapdh	Forward	TGCCACTCAGAAGACTGTGG
	Reverse	TTCAGCTCTGGGATGACCTT
RPL13A	Forward	ATGACAAGAAAAAGCGGATG
	Reverse	CTTTTCTGCCTGTTTCCGTA
Tbr2	Forward	TTCCGGGACAACTACGATTCA
	Reverse	ACGCCGTACCGACCTCC
Lacuna	Forward	CGGGTCCTCTCAAGTCAGTC

	Reverse	GTTGCTTCCACATGCTTCCT
4930593C16Rik	Forward	GTCAGGGAGGCCGTCTAGTA
	Reverse	ACCTCCGTACCCATCACCTC
9130024F11Rik	Forward	ATCTGATGATGCGATGCGATGGTTG
	Reverse	GACACACGCCAGTGAGGTTA
Ariel	Forward	CACGCGGTGATGTCTTACTG
	Reverse	AAGACACAGGCGGGTGAGC
0610009E02Rik	Forward	GCTGGAGCCTGCCCGGCGCA
	Reverse	AGTATTTATTTATGTACTTT
TCONS_00025370	Forward	CGAGGAACAGCATGGAGTCT
	Reverse	CTTCTGTGGGTGTTCCTGGT
Prox1	Forward	AAAGTCAAATGTACTCCGCAAGC
	Reverse	CTGGGAAATTATGGTTGCTCCT
Notch1	Forward	GCTGCCTCTTTGATGGCTTCGA
	Reverse	CACATTCGGCACTGTTACAGCC
Cux2	Forward	GCGGCGTTCCTGAGTGTTTAT
	Reverse	CTGGCAGGTGGTTACCGTT
Satb2	Forward	GGAGAACGACAGCGAGGAA
	Reverse	CCGAT GTATTGCTTTGCCTAGT
Ctnnb1	Forward	ATGGAGCCGGACAGAAAAGC
	Reverse	CTTGCCACTCAGGGAAGGA
Lockd	Forward	TTGCCCTGTGTCCTTTCTGTC
	Reverse	AAAGAGGACGCCTCAGGTTG
P27	Forward	GTTTCAGACGGTTCCCCGAA
	Reverse	TCTTAATTCGGAGCTGTTTACGTC
Golga4	Forward	GTTGAAGCACACGTCCACAC
	Reverse	AGTTCGGCTTCCACCTCTTG
Gm33450	Forward	GGAGGACGGGAAAGACTGTC
	Reverse	TTGTTGTAGGGCTGGCTCTG
U6long	Forward	GTGCTCGCTTCGGCAGCACA
	Reverse	GGAACGCTTCACGAATTTGCGTGTCAT

18s	Forward	TTGACGGAAGGGCACCAC
	Reverse	ACCACCACCCACGGAATC
7SK	Forward	TTCCCCGAATAGAGGAGGAC
	Reverse	GCCTCATTTGGATGTGTCTG

Mapping of Lacuna locus

As the Lacuna lncRNA is not yet annotated, we specified the boundaries of its three exons using appropriate primers and PCR (KAPA Taq PCR Kit). The templates were cDNAs derived from RNA of embryonic mouse cortices in embryonic days E12, E14, E16, E18 and newborns P0. Then, we performed gel electrophoresis of PCR products using the appropriate DNA ladder (Quick Load Purple 100 bp DNA Ladder, #NO551G, New England Biolabs).

Primers that were used for PCR are presented in the following table:

Forward Primers	
1	CTGGCACTGAGTACTCTGGGGACCCAAC
2	ACTCTGGGGACCCAACTTTT
3	CGGGTCCTCTCAAGTCAGTC
4	AAATCTCCACCGGGTGAAAG
Reverse Primers	
5	GTGGGCTTCATTTCTTCAGC
6	GTTGCTTCCACATGCTTCCT
7	GTCTATTTCAAGTCTTGTATATTTTTGCACCG

Subcellular fractionation

Neurospheres were cultured in full medium plus growth factors and were harvested in passage 2 [116,119]. Subcellular fractionation was performed with PARIS Kit Protein and RNA isolation system (Ambion, AM1921). Nuclear and cytoplasmic samples were obtained and then, we performed RNA isolation, cDNA synthesis and real time RT-qPCR analysis. Success of fractionation and normalization of measured values were achieved by using Gapdh, U6long, 18s and 7sk primers.

Immunofluorescence

For the cell immunostaining experiments, primary cells were cultured onto poly-L-lysine (Sigma) coated coverslips in 24-well plates. After electroporation, they were cultured for 3-5 days and then, fixed on the coverslips with 4% PFA. The coverslips were blocked with 5% FBS in PBS 1x containing 0.3% triton X-100 for 2 hours at room temperature (RT). Next, they were incubated with primary antibodies at 4° C overnight followed by secondary antibodies for 2 hours at RT. Finally, they were incubated with DAPI diluted in 1X PBS for 10min at RT followed by mounting with MOWIOL. The primary antibodies in the immunofluorescence were rabbit anti-phospho-Histone 3 (Abcam, Ab5176) (1:600 dilution), anti-BrdU (Abcam, 6326) (1:400 dilution), rabbit anti-cleaved caspase 3 (Cell Signaling, 9661) (1:800 dilution), mouse Tuj1/anti-beta III tubulin (Covance, MMS-435P-250) (1:1000 dilution), anti-GFAP (Abcam, 4674) (1:1500 dilution), rabbit anti-Tbr2 (Abcam, Ab23345) (1:1000 dilution), goat anti-Olig2 (1:400 dilution), mouse anti-neuN (Millipore, MAB337) (1:200 dilution), chicken anti-GFP (Abcam, Ab13790), chicken anti-mCherry (Novus, NBP2-25158) (1:1000 dilution). The secondary antibodies were donkey anti-Rabbit 488 (AlexaFluor), donkey anti-Mouse 568 (AlexaFluor), donkey anti-Rabbit 647 (AlexaFluor), donkey anti-Chicken 488 (AlexaFluor), donkey anti-Rat 488 (AlexaFluor).

In Situ Hybridization on Cryosections

Mouse embryonic brains of various developmental stages were washed in 4% PFA for 4 hours and left o/n in 30% sucrose in PBS. Then, the tissue was embedded in OCT, sectioned transversely at 12µm and collected on super-frost slides. Non-radioactive *in situ* hybridization on cryosections were carried out as previously described [116,117]. The RNA probes complementary to Lacuna, 4930593C16Rik, 9130024F11Rik lncRNAs were prepared and labeled with digoxigenin.

Statistical analysis

All experimental designs are explained in each part of the section “materials and methods”, respectively. The normal distribution of values was verified with the Shapiro–Wilk normality test using IBM SPSS Statistics for Windows, Version 20.0. To ensure the reproducibility of results, all experiments were performed independently three to four times as indicated in each figure legend. For statistical analysis all measurements and experimental values from independent experiments were estimated with two-tailed Student’s t-test or two-way ANOVA. All the results are shown as mean ± SD. The exact P values are described in each figure legend. P values < 0.05 are considered statistically significant. All analyses were done using Microsoft Excel 2013 and GraphPad Prism 8.

Results

Screening for lncRNA expression in mouse developing cortex

Preliminary data of our group extracted from an RNA-seq analysis of mouse embryonic CNS (E12.5) revealed a plethora of lncRNAs that are expressed in this tissue during development. Many of these lncRNAs were located in the genome in close proximity to protein-coding genes. This observation was in accordance with the hypothesis of the literature at that time that lncRNAs may act *in cis* affecting the expression of their neighboring protein-coding genes. Moreover, it was thought that brain development was mainly based on the expression of transcription factor genes in a very finely tuned pattern. Consequently, by comparison of the RNA-seq data and data from ENCODE and NONCODE, we identified lncRNAs that are expressed during mouse brain development and also that they are located in the genome in close proximity to transcription factor genes with established roles in neural development. With these data, we generated a list of 116 pairs of lncRNAs that are expressed in mouse embryonic brain and their neighboring genes encoding for neurodevelopmentally important transcription factors (Table 1). Our main hypothesis here is that a subset of these lncRNAs may be able to regulate their neighboring protein coding genes with established roles in neural development, being therefore able to contribute to the molecular mechanisms that control neural cell proliferation, differentiation, specification and/or patterning.

To address this hypothesis, we initially investigated the expression pattern of these lncRNAs (starting from 20 selected pairs) in mouse embryonic telencephalon throughout development (E12, E14, E16, E18, P0) with real-time RT-qPCR assays. Most of them exhibit a very low expression or remain constitutively active in all development stages, without any significant changes during major developmental transitions, e.g. from proliferation to increased neurogenesis or from neurogenesis to astrogliogenesis. These observations are in agreement with previous reports suggesting that a large number of lncRNAs are expressed in very low levels with no significant contribution to cellular phenomena [120,121]. However, there is also an increasing body of literature, especially for the cellular context of the nervous system, supporting the opposite hypothesis that a subset of lncRNAs is highly expressed and mediate critical functions in neural cells [12,122,123]. Consistent to this notion, we identified a small number of lncRNAs that have interesting expression profiles during development and intriguingly, they exhibit similar or opposite expression patterns in relation to their neighboring transcription factor genes (Figure 1). In detail, we studied the expression profiles of the following lncRNA – Transcription factor gene pairs: *0610009E02Rik* and *Notch1* (Figure 1a), *Ariel* (*AK1421611*) and *Prox1* (Figure 1b), *Lacuna*

(TCONS_00034309) and *Tbr2/Eomes* (Figure 1c), *9130024F11Rik* and *Satb2* (Figure 1d), *TCONS_00025370* and *Cux2* (Figure 1e), *4930593C16Rik* and *Ctnnb1* (Figure 1f). These preliminary data may indicate a possible involvement of the identified lncRNAs in critical neurodevelopmental transitions via cooperative or antagonistic actions with the transcription factor genes.

Table 1

Genes encoding for TFs	LncRNAs	Genes encoding for TFs	LncRNAs
Ascl4	AK018959	NFIB	AK081607
Atoh7	AK005214	NFIx	AK168184
Crebbp	4930455F16Rik	NFkb2	AK029443
Crx	Crx-OS	Ngn1	AK016084
Ctnnb1 (β-catenin)	4930593C16Rik	Nkx2.1	BC064451
Cux2	TCONS_00025370	Nkx2.2	Nkx2.2-AS
Cux2	AK187608	Notch1	0610009E02Rik
Dll1	00013250	NR2F1	AK051417
Dll4	Gm14207	NR2F1	A830082K12Rik
Dlx1	Dlx1as	NR2F2	AK135306
Dlx4	A730090H04Rik	NR3C2	Gm10649
Dlx6	Dlx6-AS1	NR4a2	BB557941
Dlx6	Dlx6-AS2	Nr5a2	AK178198
Emx2	Emx2os	Nr5a2	AK145521
Evx1	5730457N03Rik	Numbl	AK042797
Ezh2	00027084	Otx2	Otx2os
FezF1	FezF1AS1	Pax2	AK006641
FezF1	AK086573	Pax6	AK044354
Foxa2	AK156045	Pbx3	AK138624
FoxC1	00007952	Pou4F1	AK084042
FoxG1	AK158887	PPARd	AK033897
FoxG1	3110039M20Rik	PPARd	AK007468
FoxO3	AK008417	Prox1	Ariel (AK142161)
Gata1	S57880	Ptf1a	AK053418
Gata2	AK137172	RAR-a	AK031732
Gata3	4930412013R	RAR-b	AK052306
Gata4	AK031341	RBPjK	AK164362
Gata6	AK033147	RORa	00034222
Gata6	AK003136	RORb	00016088
Gbx2	D130058E05Rik	Runx1	AK131747
Gli1	AK157048	Satb2	9130024F11Rik
Gli2	AK054469	Six1	AK035085
Gli3	AK135998	Six3	Six3-AS1
Hand2	Hand2-AS	Six3	Six3-OS1
Hmx1	E130018O15Rik	Six6	4930447C04Rik
Id4	00008257	Smad5	00007996
Insm1	4930529M08Rik	Smad6	00034198
Irx2	Gm20554	Smad7	00015225
Jun	00023073	Sox1	Gm5607
Lef1	Lef1-AS1	Sox10	GM10863
Lhx1	Lhx1-OS	Sox2	Sox2-OT
Lhx3	AK035055	Sox3	TCONS_00035992
Lhx8	AI606473	Sox5	00027474
Lmx1b	C130021I20	Sox21	AK039417
Lxrb (Nr1h2)	AK184603	Sox8	AK079380
Maml1	00003937	Sox9	BC006965
Mef2c	00008122	STAT5b	AK088966
Meis1	AK144295	Tbr2 (eomes)	TCONS_00034309
Meis2	AK012325	Tgif2	5430405H02Rik
Meis2	AK144367	THRA	AK165172
Meis2	AK144485	THRB	AK088911
Msx1	Msx1as	Tle1	00022626
MycN	MycN-os	Tlx3	00003913
Myt1L	AK138505	WT1	AK033304
NeuroD1	00017302	WT1	AI314831
NFATc1	AK155068	Zeb1	AK041408
NFATc4	AK014164	Zeb1	Gm10125
NFI	E130114P18Rik	Zeb2	Zeb2-OS

Table 1. List of pairs of lncRNAs – Transcription factor genes. Pairs of lncRNAs that are expressed in mouse embryonic brain during development (green) with their neighboring protein coding genes encoding for transcription factors with established roles in brain development (blue).

Figure 1

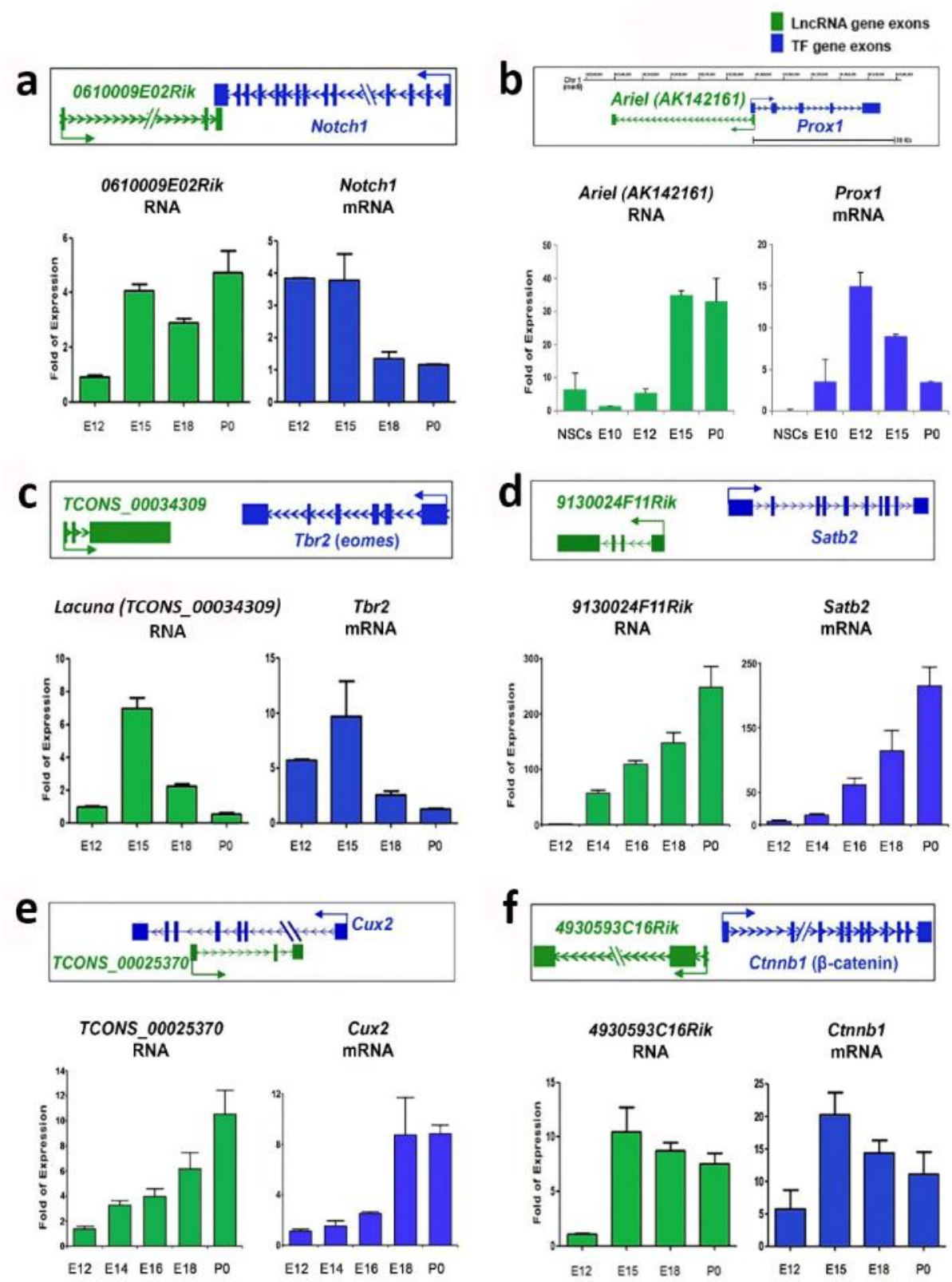

LncRNA gene exons
TF gene exons
a
0610009E02Rik
Notch1
b
Ariel (AK142161)
Prox1
0610009E02Rik RNA
Notch1 mRNA
Ariel (AK142161) RNA
Prox1 mRNA
Fold of Expression
E12 E15 E18 P0
NSCs E10 E12 E15 P0
c
TCONS_00034309
Tbr2 (eomes)
d
9130024F11Rik
Satb2
Lacuna (TCONS_00034309) RNA
Tbr2 mRNA
9130024F11Rik RNA
Satb2 mRNA
E12 E14 E16 E18 P0
e
Cux2
TCONS_00025370
f
4930593C16Rik
Ctnnb1 (β-catenin)
TCONS_00025370 RNA
Cux2 mRNA
4930593C16Rik RNA
Ctnnb1 mRNA

Figure 1. Expression profiles of selected lncRNAs – Transcription factor genes during mouse brain development. In each box, corresponding genetic locus is shown schematically. LncRNAs are shown in green and transcription factor genes in blue. RNA samples were collected from mouse embryonic telencephalon in each developmental stage, as indicated. **(a)** RNA levels of *0610009E02Rik* and *Notch1* **(b)** RNA levels of *Ariel* (*AK1421611*) and *Prox1* **(c)** RNA levels of *Lacuna* (TCONS_00034309) and *Tbr2/Eomes* **(d)** RNA levels of *9130024F11Rik* and *Satb2* **(e)** RNA levels of *TCONS_00025370* and *Cux2* **(f)** RNA levels of *4930593C16Rik* and *Ctnnb1*.

Investigation of selected lncRNA – Transcription Factor gene pairs

AK1421611, named by our group as *Ariel*, has already been investigated extensively by other members of our group and found to be necessary and sufficient for proper differentiation of NSCs. In particular, *Ariel* affects astrogliogenesis via direct and negative regulation of the Prox1 gene, which is encoding for a transcription factor with critical role in the opposite effect, induction of neurogenesis (manuscript under preparation). Prompted by this example, we decided to investigate the rest of our selected pairs. To this end, we performed a rapid experimental screen to choose the best candidate lncRNA for further analysis. In particular, we undertook *in situ* hybridization experiments on embryonic mouse telencephalon and overexpression studies of the selected lncRNA in the Neuro2A (N2A) cells, a fast-growing, easily transfected mouse neuroblastoma cell line.

For *4930593C16Rik* (which is next to *Ctnnb1* gene), *in situ* hybridizations showed a localization towards the cortical plate of the developing mouse brain (Figure 2a). Upon its overexpression in N2A cells, its neighboring *Ctnnb1* gene was upregulated significantly (Figure 2b), but this result could not be verified with Western blot nor could it be replicated in primary NSCs. For *9130024F11Rik* (which is next to *Satb2* gene), *in situ* hybridizations showed a robust expression in the cortical plate (Figure 2c), but upon overexpression of the lncRNA, its neighboring *Satb2* gene is not affected (Figure 2d). Other lncRNAs (like *TCONS_00025370* and *0610009E02Rik*) could not be neither detected with *in situ* hybridization nor properly overexpressed in N2A cells.

Figure 2

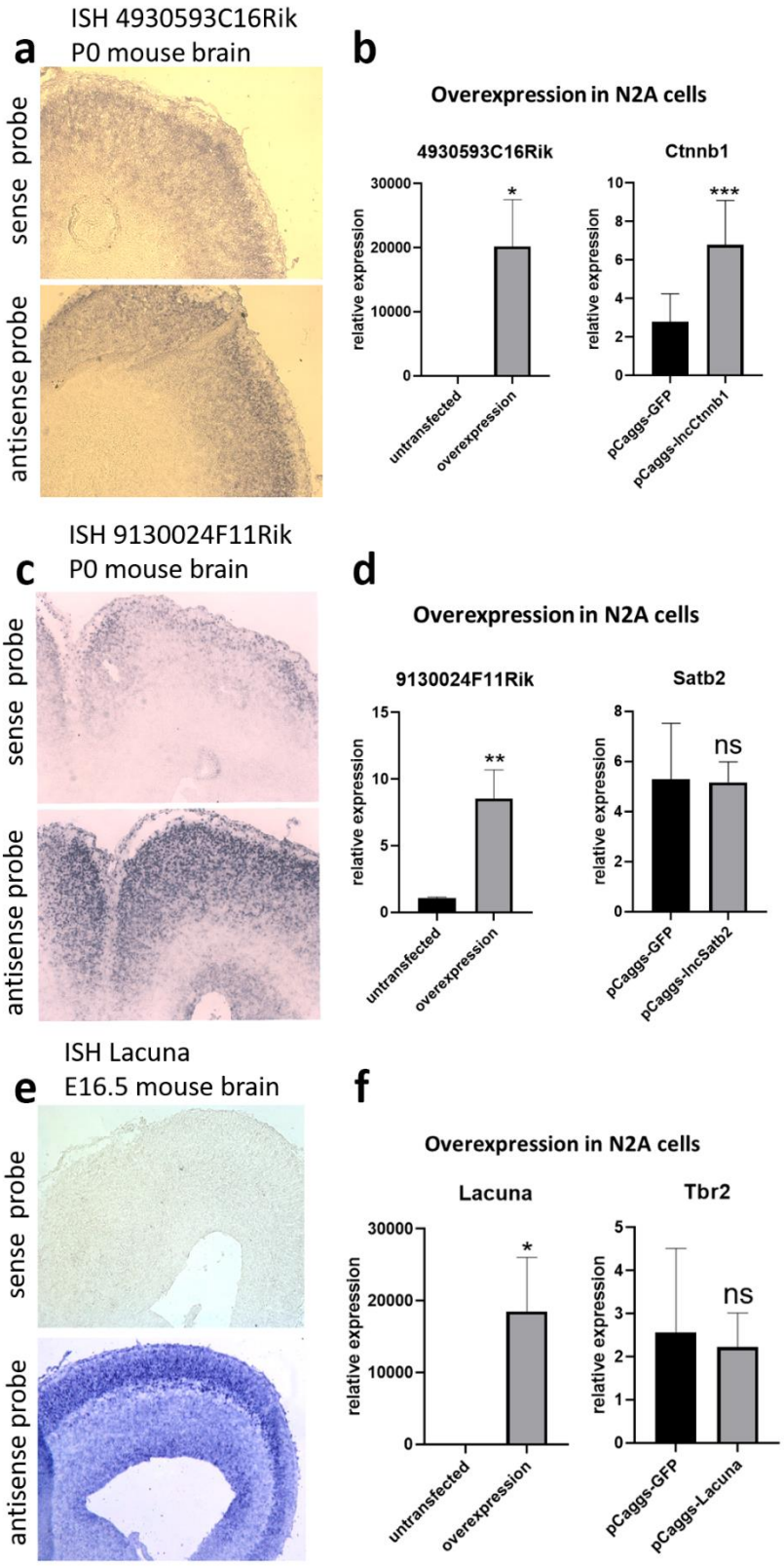

a
ISH 4930593C16Rik
P0 mouse brain
sense probe
antisense probe
b
Overexpression in N2A cells
4930593C16Rik
relative expression
30000
20000
10000
0
*
untransfected
overexpression
Ctnnb1
relative expression
10
8
6
4
2
0
***
pCaggs-GFP
pCaggs-lncCtnnb1
c
ISH 9130024F11Rik
P0 mouse brain
sense probe
antisense probe
d
Overexpression in N2A cells
9130024F11Rik
relative expression
15
10
5
0
**
untransfected
overexpression
Satb2
relative expression
8
6
4
2
0
ns
pCaggs-GFP
pCaggs-lncSatb2
e
ISH Lacuna
E16.5 mouse brain
sense probe
antisense probe
f
Overexpression in N2A cells
Lacuna
relative expression
30000
20000
10000
0
*
untransfected
overexpression
Tbr2
relative expression
5
4
3
2
1
0
ns
pCaggs-GFP
pCaggs-Lacuna

Figure 2. Expression pattern of *4930593C16Rik*, *9130024F11Rik* and *Lacuna* in mouse embryonic brain and overexpression on N2A cells. (a) *In situ* hybridization of *4930593C16Rik* on P0 mouse embryonic brain **(b)** RNA levels of *4930593C16Rik* and *Ctnnb1* upon overexpression of *4930593C16Rik* in N2A cells **(c)** *In situ* hybridization of *9130024F11Rik* on P0 mouse embryonic brain **(d)** RNA levels of *9130024F11Rik* and *Satb2* upon overexpression of *9130024F11Rik* in N2A cells **(e)** *In situ* hybridization of *Lacuna* on E16.5 mouse embryonic brain **(f)** RNA levels of *Lacuna* and *Tbr2* upon overexpression of *Lacuna* in N2A cells.

Lacuna lncRNA is expressed in the developing mouse brain cortex

TCONS_00034309, named by our group as *Lacuna*, did not seem to affect the expression of its neighboring *Tbr2* gene (Figure 2f) upon overexpression in N2A cells, but *in situ* hybridizations revealed an intriguing expression pattern. Indeed, *in situ* hybridization (Figure 3d) and real time RT-qPCR assays (Figure 3b-c) showed that *Lacuna* expression peaks at E14 – E16 in the developing mouse cortex and declines dramatically perinatally and postnatally, following the expression pattern of its neighboring transcription factor gene, *Tbr2/Eomes*. More specifically, *Lacuna* expression is apparent in the cortical plate of the cortex at E16.5, whereas at E14.5 we can only observe a weak expression in this area (Figure 3d).

As *Lacuna* is not yet annotated, we verified its RNA sequence and its exon-to-exon junctions. To do that, we designed multiple specific primers to verify the existence of three concrete exons in the *Lacuna* lncRNA of 1661 nt in all developmental stages. We showed that *Lacuna* is not subjected to alternative splicing in mouse developing brain, as there are standard exon-to-exon junctions and none of the exons is skipped during transcription (Figure 4a-b). We were also able to find the same RNA sequence in the NONCODE RNA Database (NONMMUT071331), where it was shown that Lacuna was detected in mouse adult heart, liver, lung, spleen, thymus and higher in adult hippocampus (Figure 4c), where adult neural stem and progenitor cells reside.

Furthermore, to define the subcellular localization of *Lacuna*, we performed subcellular fractionation of NSCs in conjunction with real time RT-qPCR. Accordingly, we were able to show that *Lacuna* is localized both in the cytosol and the nucleus (Figure 3e). In agreement, analysis of higher magnification images from *in situ* hybridization experiments on E16.5 mouse embryonic cortex (Figure 3f) nicely confirmed these data. Therefore, *Lacuna* RNA is found both at cytoplasm and nucleus, indicating a molecule that may exert nuclear and/or cytoplasmic roles.

Figure 3

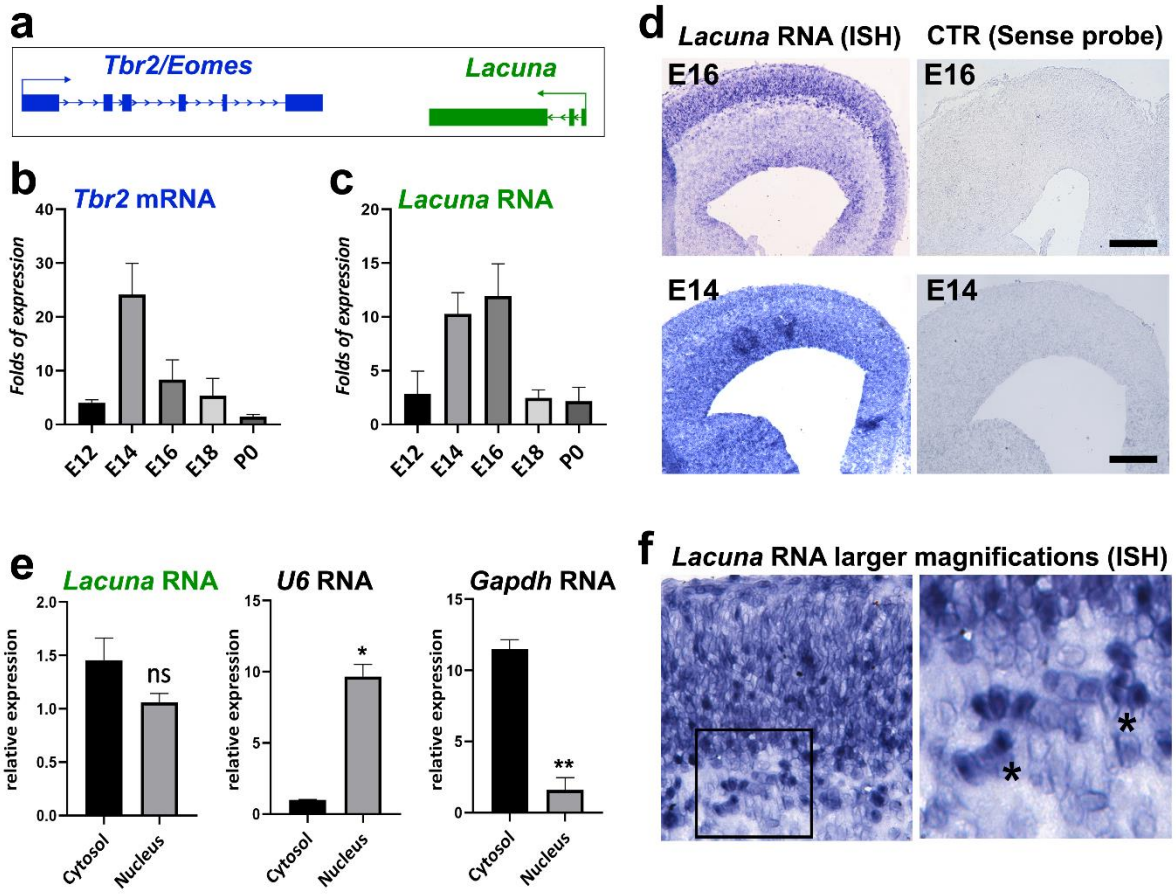


Figure 3. *Lacuna* is expressed in mouse brain during embryonic development. (a) Schematic representation of *Tbr2/Eomes* and *Lacuna* locus **(b)** mRNA levels of *Tbr2/Eomes* during mouse embryonic brain development **(c)** RNA levels of *Lacuna* during mouse embryonic brain development **(d)** *In situ* hybridization of *Lacuna* on E16 and E14 mouse embryonic brain with the corresponding controls **(e)** Subcellular fractionation of NSCs and RNA levels of *Lacuna* in each subcellular compartment. mRNA levels of *U6* and *Gapdh* were used to verify the fractionation of cells ($p<0.01$, n=3) **(f)** *In situ* hybridization of *Lacuna* on E16.5 mouse embryonic brain. Zoomed square indicates with asterisks the localization of *Lacuna* both in cytosol and nucleus.

Figure 4

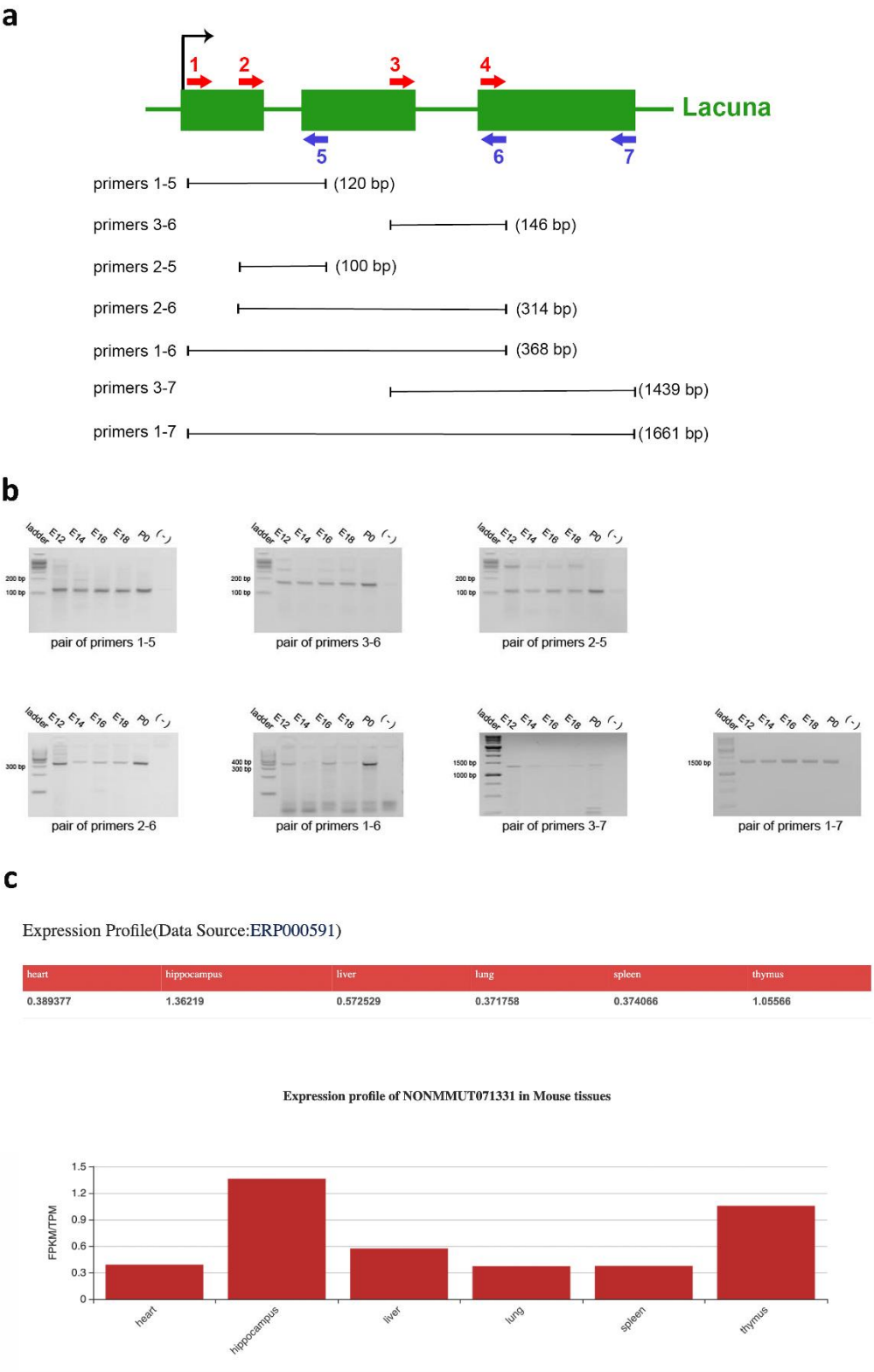

a
1
2
3
4
Lacuna
5
6
7
primers 1-5 (120 bp)
primers 3-6 (146 bp)
primers 2-5 (100 bp)
primers 2-6 (314 bp)
primers 1-6 (368 bp)
primers 3-7 (1439 bp)
primers 1-7 (1661 bp)
b
ladder E12 E14 E16 E18 P0 (-)
200 bp
100 bp
pair of primers 1-5
pair of primers 3-6
pair of primers 2-5
300 bp
pair of primers 2-6
400 bp
300 bp
pair of primers 1-6
1500 bp
1000 bp
pair of primers 3-7
1500 bp
pair of primers 1-7
c
Expression Profile(Data Source:ERP000591)
heart
hippocampus
liver
lung
spleen
thymus
0.389377
1.36219
0.572529
0.371758
0.374066
1.05566
Expression profile of NONMMUT071331 in Mouse tissues
FPKM/TPM
1.5
1.2
0.9
0.6
0.3
0
heart
hippocampus
liver
lung
spleen
thymus

Figure 4. Mapping of *Lacuna* locus by RT-PCR assays. (a) Scheme of the experimental design. Pairs of specific primers were used to map different exons of *Lacuna* with PCR. **(b)** PCR products of each pair were analyzed by agarose gel electrophoresis. PCR template was cDNA (random primers) produced by RNA extracted from mouse embryonic telencephalon of each developmental stage (E12, E14, E16, E18, P0). DNA Ladder: Quick Load Purple 100bp DNA Ladder **(c)** Lacuna was found in NONCODE Genome Database as NONMMUT071331 to be expressed in various mouse adult tissues, including adult mouse hippocampus.

Lacuna overexpression inhibits neuronal differentiation of NSCs

To gain further insights into the functional role of *Lacuna* in NSCs, we first studied the effect of its overexpression on *ex vivo* cultured NSCs. Specifically, we constructed plasmids that were sufficient to overexpress *Lacuna* and GFP under the CAGG promoter, which works best with mammalian cells and lncRNAs [116,117,119]. A mixture of two plasmids, pCAGGs-*Lacuna* and pCAGGs-GFP (experimental condition) or pCAGGS empty and pCAGGS-GFP (control condition), was used to transfect NSCs with Amaxa electroporation technique (Figure 5a). In addition, by using Amaxa electroporation, we have previously established and reported methodologies to perform gain- and loss-of-function experiments in embryonic NSCs (isolated from developing mouse telencephalon and ex vivo cultured in defined media) as well as to extensively investigate the contribution of genes and molecular players in proliferation, differentiation, specification, and maturation of neural cells [116,117,119].

Remarkably, transfection of pCAGGs-*Lacuna* caused a significant increase in Nestin, a marker of neural cell stemness (Figure 5d-e), although proliferation as well as apoptosis were found unaffected (Figure 5b-c, 5f-g). In accordance, in *Lacuna* overexpressing NSCs, the ability to produce βIII-tubulin+ neurons (Figure 6a-b) and NeuN+ neurons (Figure 6c-d) under differentiation conditions [without growth factors (GF)] was found significantly reduced as compared to the control transfections. Astrogliogenic differentiation (GFAP marker) seems to remain unaffected under *Lacuna* overexpression (Figure 6g-h). However, upon *Lacuna* overexpression, we found significantly increased population of Olig2+ cells (Figure 6e-f) and as the NSCs are derived from E14.5 mouse embryonic cortex, we assume that this extra population corresponds to Olig2-expressing neural progenitor cells that are not able to differentiate into neurons. Therefore, these observations suggest that *Lacuna* is sufficient to exert a mild, yet statistically significant, effect on the ability of NSCs to generate neurons without affecting astrogliogenic or proliferative capacities of these cells.

Figure 5

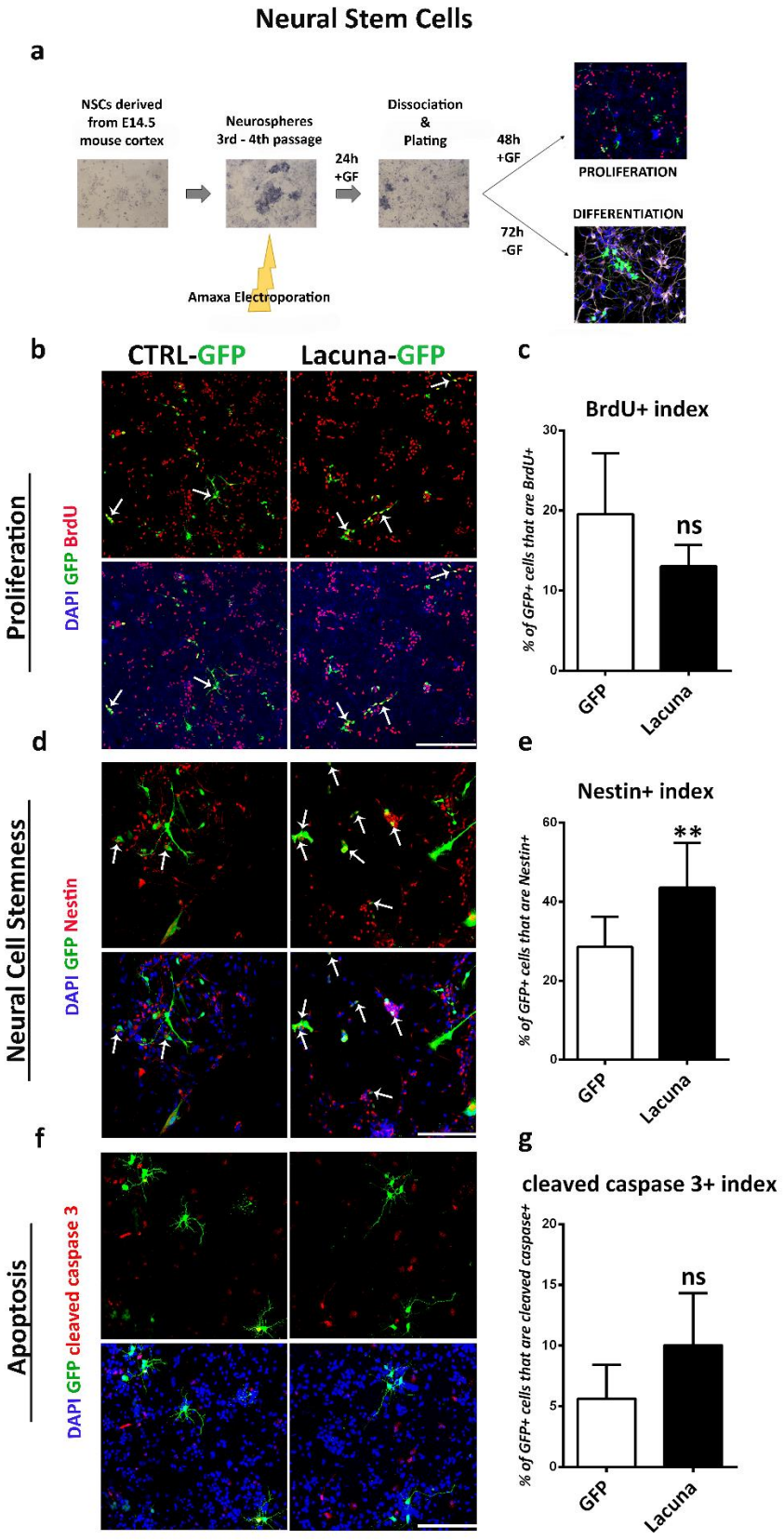

Neural Stem Cells
a
NSCs derived from E14.5 mouse cortex
Neurospheres 3rd - 4th passage
Dissociation & Plating
24h +GF
48h +GF
PROLIFERATION
72h -GF
DIFFERENTIATION
Amaxa Electroporation
b
CTRL-GFP
Lacuna-GFP
Proliferation
DAPI GFP BrdU
c
BrdU+ index
% of GFP+ cells that are BrdU+
ns
GFP
Lacuna
d
Neural Cell Stemness
DAPI GFP Nestin
e
Nestin+ index
% of GFP+ cells that are Nestin+
**
f
Apoptosis
DAPI GFP cleaved caspase 3
g
cleaved caspase 3+ index
% of GFP+ cells that are cleaved caspase+

Figure 5. *Lacuna* overexpression affects stemness but not proliferation nor apoptosis of mouse Neural Stem Cells. (a) Scheme of experimental strategy. NSCs are derived from E14.5 mouse cortices and they are cultured appropriately to form neurospheres over 3 – 4 passages. Neurospheres are transfected with plasmids of choice and then, they are dissociated and plated. In the presence of growth factors, NSCs proliferate, whereas without growth factors, they differentiate to generate neurons and astrocytes **(b)** *Lacuna-GFP* and *Control-GFP* transfected Neural Stem cells were treated with BrdU for 2h and then fixed and stained with anti-BrdU antibody (red), anti-GFP antibody (green) and 4, 6-diamidino-2-phenylindole (DAPI). Scale bar: 0-250 μM **(c)** Quantification of BrdU incorporation in transgene positive mouse Neural Stem cells (GFP: 19,55 ± 3,104%, Lacuna: 13,06 ± 1,098%, $p>0.05$). **(d)** *Lacuna-GFP* and *Control-GFP* transfected mouse Neural Stem cells were immunostained for Nestin (red), GFP (green) and labeled with DAPI. Scale bar: 0-100 μM **(e)** Quantification of Nestin positive cells in transgene positive mouse Neural Stem cells (GFP: 28,59 ± 2,691%, Lacuna: 43,55 ± 4,019%, $p<0.01$) **(f)** *Lacuna-GFP* and *Control-GFP* transfected mouse Neural Stem cells were immunostained for cleaved caspase 3 (red), and GFP (green) and labeled with DAPI. Scale bar: 250 μM **(g)** Quantification of cleaved caspase 3 positive cells in transgene positive mouse Neural Stem cells (GFP: 5,629 ± 1,409%, Lacuna: 10,05 ± 2,144%, $p>0.05$). For all cases, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, n=3

Figure 6

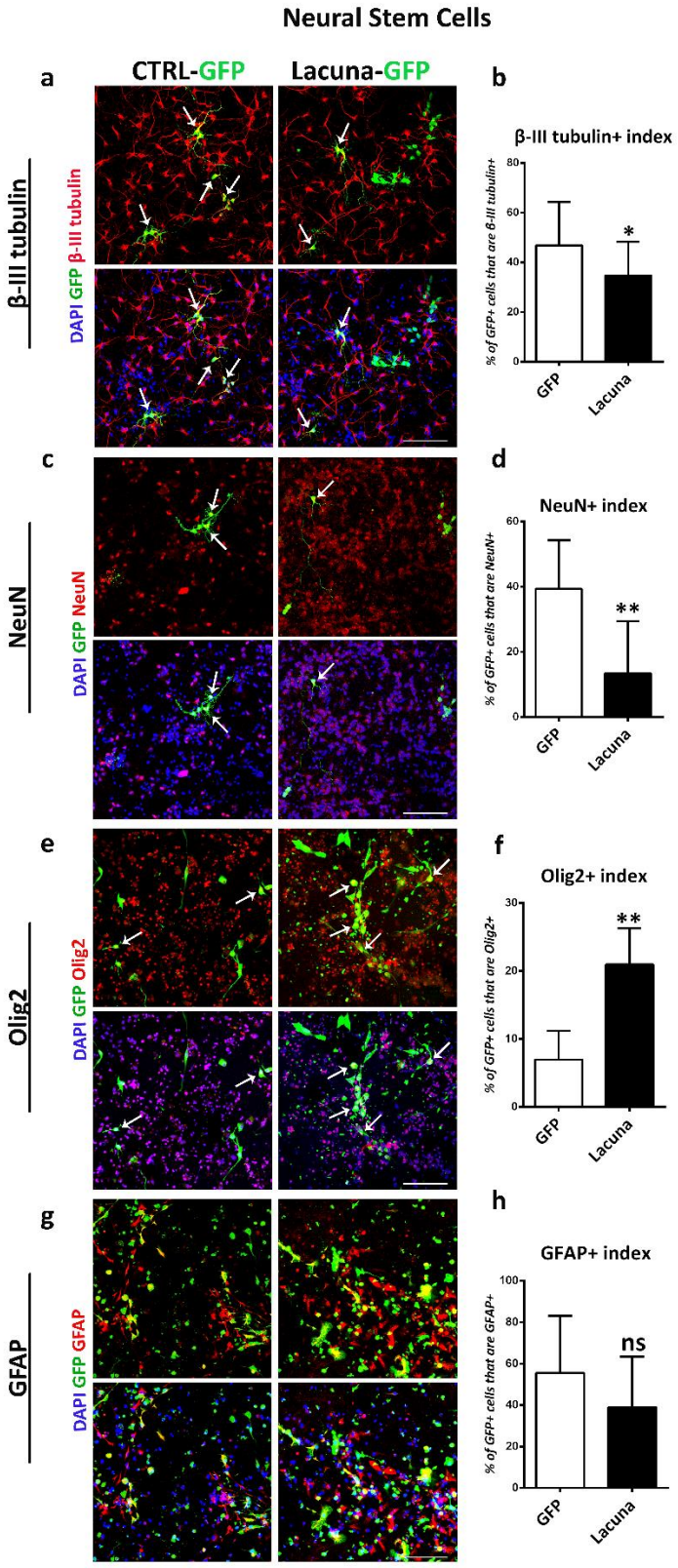

Neural Stem Cells
CTRL-GFP
Lacuna-GFP
a
b
β-III tubulin
DAPI GFP β-III tubulin
β-III tubulin+ index
% of GFP+ cells that are β-III tubulin+
*
GFP
Lacuna
c
d
NeuN
DAPI GFP NeuN
NeuN+ index
% of GFP+ cells that are NeuN+
**
e
f
Olig2
DAPI GFP Olig2
Olig2+ index
% of GFP+ cells that are Olig2+
**
g
h
GFAP
DAPI GFP GFAP
GFAP+ index
% of GFP+ cells that are GFAP+
ns

Figure 6. *Lacuna* overexpression inhibits proper neuronal differentiation of mouse Neural Stem Cells. **(a)** *Lacuna-GFP* and *Control-GFP* transfected mouse Neural Stem cells were immunostained for β-III tubulin (red) and GFP (green) and labeled with DAPI. Scale bar: 100 μM **(b)** Quantification of β-III tubulin positive cells in transgene positive mouse Neural Stem cells (GFP: 46,82 ± 4,154%, Lacuna: 34,73 ± 3,399%, $p<0.05$). **(c)** *Lacuna-GFP* and *Control-GFP* transfected mouse Neural Stem cells were immunostained for NeuN (red), GFP (green) and labeled with DAPI. Scale bar: 100 μM **(d)** Quantification of NeuN positive cells in transgene positive mouse Neural Stem cells (GFP: 39,35 ± 4,721%, Lacuna: 13,37 ± 5,353, $p<0.01$) **(e)** *Lacuna-GFP* and *Control-GFP* transfected mouse Neural Stem cells were immunostained for Olig2 (red), GFP (green) and labeled with DAPI. Scale bar: 100 μM **(f)** Quantification of Olig2 positive cells in transgene positive mouse Neural Stem cells (GFP: 6,961 ± 1,905%, Lacuna: 21,00 ± 2,387%, $p<0.05$) **(g)** *Lacuna-GFP* and *Control-GFP* transfected mouse Neural Stem cells were immunostained for GFAP (red), GFP (green) and labeled with DAPI. Scale bar: 100 Mm **(h)** Quantification of GFAP positive cells in transgene positive mouse Neural Stem cells (GFP: 55,50 ± 7,372%, Lacuna: 38,90 ± 6,521%, $p>0.05$) For all cases, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, n=3

Lacuna knockdown promotes differentiation in NSCs

To further investigate the involvement of *Lacuna* in NSCs fate decision, we assessed whether *Lacuna* is necessary for NSCs differentiation by performing knockdown experiments using a CRISPR-dCas9-KRAB effector system. This system is highly effective and specific in knocking down lncRNAs expression, but also it leaves DNA intact [124,125], meaning that there are no changes at the level of DNA sequence (Figure 7a), as is the case with the traditional CRISPR-Cas9 methodology. This feature will help us to elucidate whether a lncRNA, in this case *Lacuna*, acts via its RNA or via its DNA sequence elements. It has been previously shown that deletion of lncRNAs genomic loci led to significant effects on cellular functions, only to be subsequently proved by follow-up studies that the causal factor lies on the DNA (e.g regulatory DNA elements) and not on the RNA [126]. To achieve the CRISPR-dCas9-KRAB-mediated knockdown of *Lacuna* RNA expression, we utilized 3 different guide RNAs (sgRNAs). All of them have been designed in such a way (GenCRISPR gRNA Design Tool) to target the first exon of *Lacuna* gene, as this is how KRAB inhibitor exerts best its properties. Thus, we showed that all three of them are able to downregulate the expression of *Lacuna* RNA (Figure 7b), so we continued our studies with the gRNA that had the strongest effect (75% repression).

Conversely to the overexpression studies, *Lacuna* knockdown in primary NSCs resulted in a statistically significant increase of β-III tubulin+ neurons (Figure 8a-b) and NeuN+ neurons (Figure 8c-d), but also of GFAP+ astrocytes (Figure 8g-h), as shown by immunofluorescent experiments on NSCs cultures in the absence of GFs. The Olig2+ population was found decreased (Figure 8e-f), hence exhibiting an opposite effect than that of *Lacuna* overexpression condition. Again, apoptosis and proliferation are not affected upon *Lacuna* knockdown in NSCs (Figure 7c-d, 7g-h), but Nestin is slightly, nevertheless significantly, decreased (Figure 7e-f). Taken together, these observations indicate that *Lacuna* RNA is critically involved in the regulation of neurogenesis in NSCs.

Figure 7

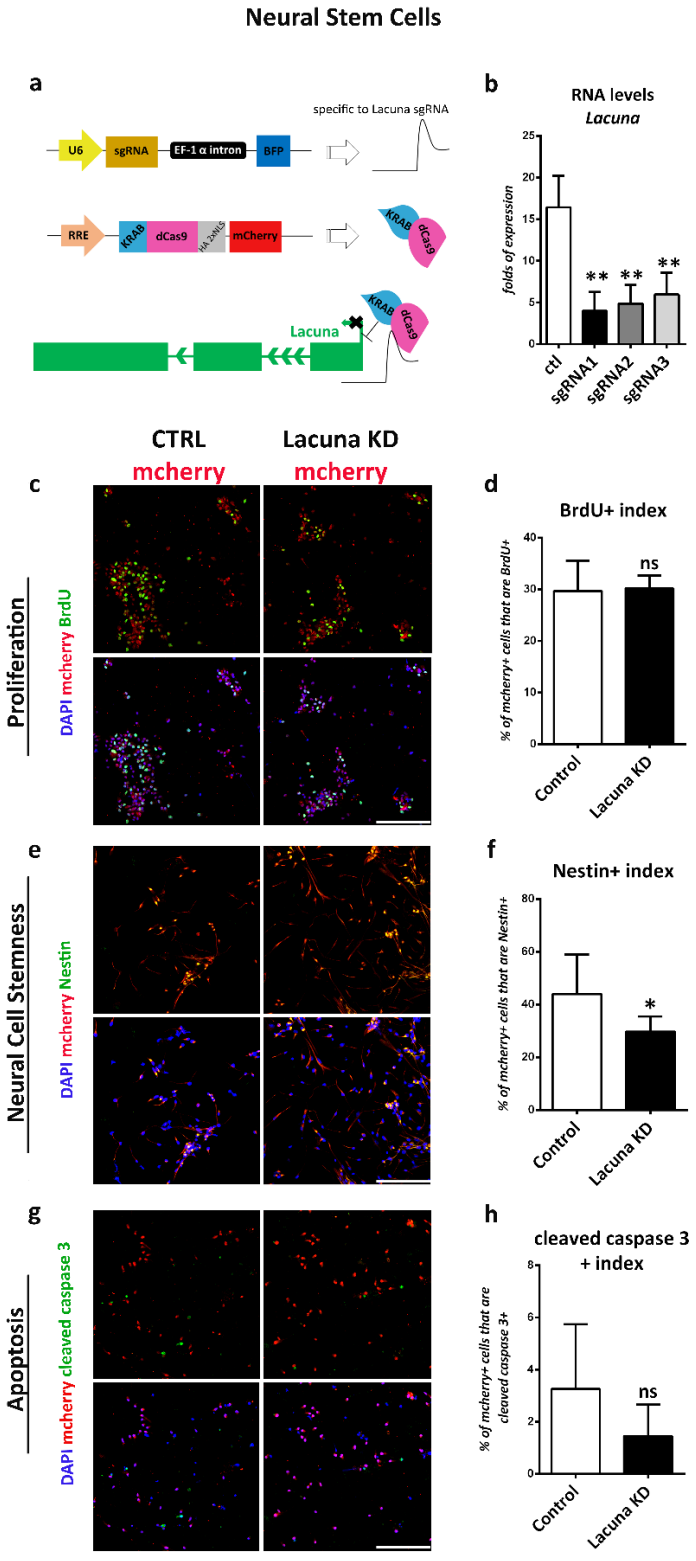

Neural Stem Cells
a
specific to Lacuna sgRNA
U6
sgRNA
EF-1 α intron
BFP
RRE
KRAB
dCas9
mCherry
Lacuna
b
RNA levels
Lacuna
folds of expression
ctl
sgRNA1
sgRNA2
sgRNA3
CTRL
mcherry
Lacuna KD
mcherry
c
Proliferation
DAPI mcherry BrdU
d
BrdU+ index
% of mcherry+ cells that are BrdU+
ns
Control
Lacuna KD
e
Neural Cell Stemness
DAPI mcherry Nestin
f
Nestin+ index
% of mcherry+ cells that are Nestin+
Control
Lacuna KD
g
Apoptosis
DAPI mcherry cleaved caspase 3
h
cleaved caspase 3 + index
% of mcherry+ cells that are cleaved caspase 3+
ns
Control
Lacuna KD

Figure 7. *Lacuna* knockdown reduces stemness but does not affect proliferation nor apoptosis of mouse Neural Stem Cells. (a) Scheme of dCas9-KRAB effector system and Lacuna knockdown strategy. The first plasmid expresses the guide RNAs that target *Lacuna*, the second plasmid expresses *dCas9-KRAB* and mCherry. When transfected together in Neural Stem cells, guide RNA recruits dCas9-KRAB fusion protein to Lacuna and inhibits its expression. In control cultures, NSCs were transfected with both plasmids, but first plasmid lacked a guide RNA sequence **(b)** Three different guide RNA sequences were used to target *Lacuna* gene. All constructs were efficient in knocking down *Lacuna* expression. We selected sgRNA1 to proceed further. **(c)** Mouse Neural Stem cells were transfected with dCas9-KRAB-mcherry and sgRNA1 targeting *Lacuna* (Lacuna KD-mcherry) or no guide RNA (CTRL-mcherry). They were treated with BrdU for 2h and then fixed and stained with anti-BrdU antibody (green), anti-mcherry (red) and 4, 6-diamidino-2-phenylindole (DAPI). Scale bar: 100 μM **(d)** Quantification of BrdU incorporation in dCas9-KRAB-mcherry positive mouse Neural Stem cells (Control: 29,67 ± 2,63%, Lacuna KD: 30,18 ± 1,115%, $p>0.05$). **(e)** Mouse Neural Stem cells were transfected with dCas9-KRAB-mcherry and sgRNA1 targeting *Lacuna* (Lacuna KD-mcherry) or no guide RNA (CTRL-mcherry). They were immunostained for Nestin (green), mcherry (red) and labeled with DAPI. Scale bar: 100 μM **(f)** Quantification of Nestin positive cells in dCas9-KRAB-mcherry positive mouse Neural Stem cells (Control: 43,99 ± 5,664%, Lacuna KD: 29,67 ± 2,176%, $p<0.05$) **(g)** Mouse Neural Stem cells were transfected with dCas9-KRAB-mcherry and sgRNA1 targeting *Lacuna* (Lacuna KD-mcherry) or no guide RNA (CTRL-mcherry). They were immunostained for cleaved caspase 3 (green), mcherry (red) and labeled with DAPI. Scale bar: 100 μM **(h)** Quantification of cleaved caspase 3 positive cells in dCas9-KRAB-mcherry positive mouse Neural Stem cells (Control: 3,261 ± 1,013%, Lacuna KD: 1,448 ± 0, 4918%, $p>0.05$) For all cases, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, n=3

Figure 8

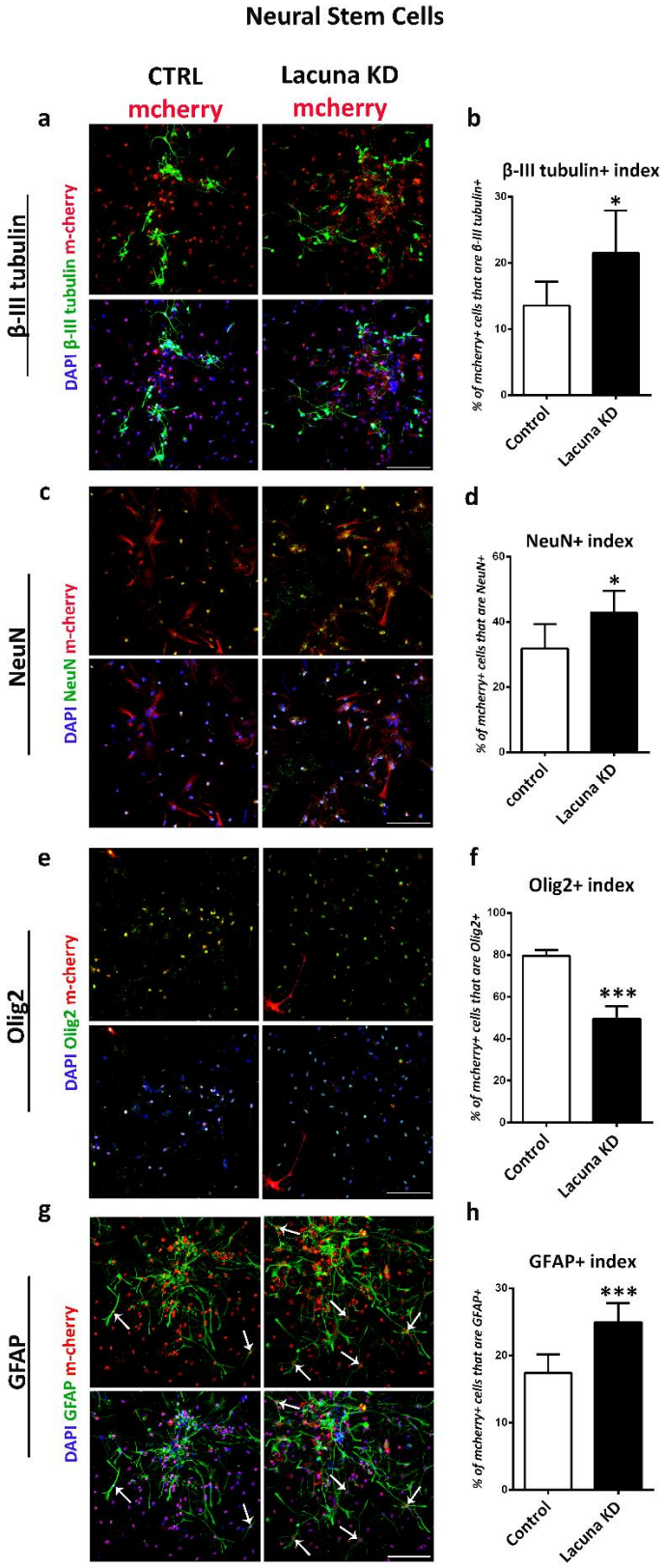

Neural Stem Cells
CTRL
mcherry
Lacuna KD
mcherry
a
b
β-III tubulin
DAPI β-III tubulin m-cherry
β-III tubulin+ index
% of mcherry+ cells that are β-III tubulin+
Control
Lacuna KD
c
d
NeuN
DAPI NeuN m-cherry
NeuN+ index
% of mcherry+ cells that are NeuN+
control
Lacuna KD
e
f
Olig2
DAPI Olig2 m-cherry
Olig2+ index
% of mcherry+ cells that are Olig2+
Control
Lacuna KD
g
h
GFAP
DAPI GFAP m-cherry
GFAP+ index
% of mcherry+ cells that are GFAP+
Control
Lacuna KD

Figure 8. *Lacuna* knockdown promotes differentiation of mouse Neural Stem Cells. (a) Mouse Neural Stem cells were transfected with dCas9-KRAB-mcherry and sgRNA1 targeting *Lacuna* (Lacuna KD-mcherry) or no guide RNA (CTRL-mcherry). They were immunostained for β-III tubulin (green), mcherry (red) and labeled with DAPI. Scale bar: 100 μM **(b)** Quantification of β-III tubulin positive cells in dCas9-KRAB-mcherry positive mouse Neural Stem cells (Control: 13,54 ± 1,481%, Lacuna KD: 21,50 ± 2,617%, $p<0.05$). **(c)** Mouse Neural Stem cells were transfected with dCas9-KRAB-mcherry and sgRNA1 targeting *Lacuna* (Lacuna KD-mcherry) or no guide RNA (CTRL-mcherry). They were immunostained for NeuN (green), mcherry (red) and labeled with DAPI. Scale bar: 100 μM **(d)** Quantification of NeuN positive cells in dCas9-KRAB-mcherry positive mouse Neural Stem cells (Control: 31,86 ± 3,062%, Lacuna KD: 42,86 ± 2,723, $p<0.05$) **(e)** Mouse Neural Stem cells were transfected with dCas9-KRAB-mcherry and sgRNA1 targeting *Lacuna* (Lacuna KD-mcherry) or no guide RNA (CTRL-mcherry). They were immunostained for Olig2 (green), mcherry (red) and labeled with DAPI. Scale bar: 0-100 μM **(f)** Quantification of Olig2 positive cells in dCas9-KRAB-mcherry positive mouse Neural Stem cells (Control: 79,59 ± 1,394%, Lacuna KD: 49,53 ± 3,023%, $p>0.001$) **(g)** Mouse Neural Stem cells were transfected with dCas9-KRAB-mcherry and sgRNA1 targeting *Lacuna* (Lacuna KD-mcherry) or no guide RNA (CTRL-mcherry). They were immunostained for GFAP (green), mcherry (red) and labeled with DAPI. Scale bar: 100 Mm **(h)** Quantification of GFAP positive cells in dCas9-KRAB-mcherry positive mouse Neural Stem cells (Control: 17,43 ± 1,124%, Lacuna KD: 24,94 ± 1,165%, $p<0.001$) For all cases, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, n=3

Lacuna is necessary for Tbr2/Eomes expression in NSCs

Next, we wanted to investigate whether the effect of *Lacuna* on NSCs is mediated through a possible action on the *Tbr2/Eomes* gene expression. Towards this direction, we examined whether knockdown of *Lacuna* affects the mRNA expression of *Tbr2/Eomes* and/or other genes in its genomic neighborhood. First, we wanted to confirm the specificity of our approach. Accordingly, we searched for possible effects on neighboring to *Lacuna* genes and specifically, on *Golga4* gene and a recently annotated non-coding RNA gene, *Gm33460* (Figure 9a). *Golga4* is approximately 16500 bp away from the 5' of *Lacuna* and it encodes one of the golgins, a family of proteins localized in the Golgi apparatus. *Gm33460* is downstream to *Lacuna* with a small common sequence shared between these two transcripts (end of 2nd exon and beginning of 3rd exon), but it continues after the RNA sequence of *Lacuna* (Figure 9a). Notably, both *Golga4* and *Gm33460* are not affected by KRAB-dCas9 that is targeted to *Lacuna* sequence, as shown by real time RT-qPCR (Figure 9d-e). These observations suggest that our knockdown approach is specific and sufficient to downregulate *Lacuna* expression, without affecting the other two genes, which are found close to *Lacuna* transcription start site (TSS).

Most importantly, upon knockdown of *Lacuna* in NSCs and under minus growth factor conditions, *Tbr2/Eomes* gene expression is downregulated, as shown by real time RT-qPCR assays (Figure 9b-c). Consistently, knockdown of *Lacuna* induced a statistically significant downregulation of the Tbr2 expression at the protein level as well, as shown with immunofluorescence experiments (Figure 9f-g). On the other hand, *Lacuna* overexpression does not seem to affect the amount of TBR2+ intermediate progenitor cells (Figure 9h-i), nor *Tbr2* gene expression at the mRNA level (Figure 9j-k). This difference between knockdown and overexpression probably indicates that *Lacuna* is able to regulate *Tbr2/Eomes* gene only *in cis*. Thus, only when we are knocking down the cis-expressed *Lacuna* gene, we are observing an effect on *Tbr2/Eomes* gene expression. Therefore, we favor a conclusion that Lacuna is positively regulating the expression of *Tbr2/Eomes in cis.*

The next question was whether this action could explain the effect of *Lacuna* on neuronal differentiation. Surprisingly, *Tbr2* exerts an opposite function in this context. In particular, *Tbr2* promotes neuronal differentiation [21,22,25,26]. Therefore, *Lacuna* mediated-regulation of *Tbr2/Eomes* gene expression could not explain its role in inhibiting neuronal differentiation. In agreement, *Lacuna* overexpression can inhibit neuronal differentiation without affecting *Tbr2/Eomes* expression. Therefore, we propose a hypothetical model where *Lacuna* exerts a Tbr2-independent effect on differentiation via a mechanistic action in the nucleus and/or in the cytoplasm. Moreover, the fact that two genes from the

same genomic locus are co-expressed with the same pattern, yet they exert opposite roles, may indicate that positive and negative effectors of a cellular phenomenon are co-regulated to fine-tune the final outcome. This hypothetical scenario may point to a new emerging paradigm in genome science, where lncRNAs are co-regulated with protein coding genes with opposite function to fine-tune the cellular action of the latter.

Figure 9

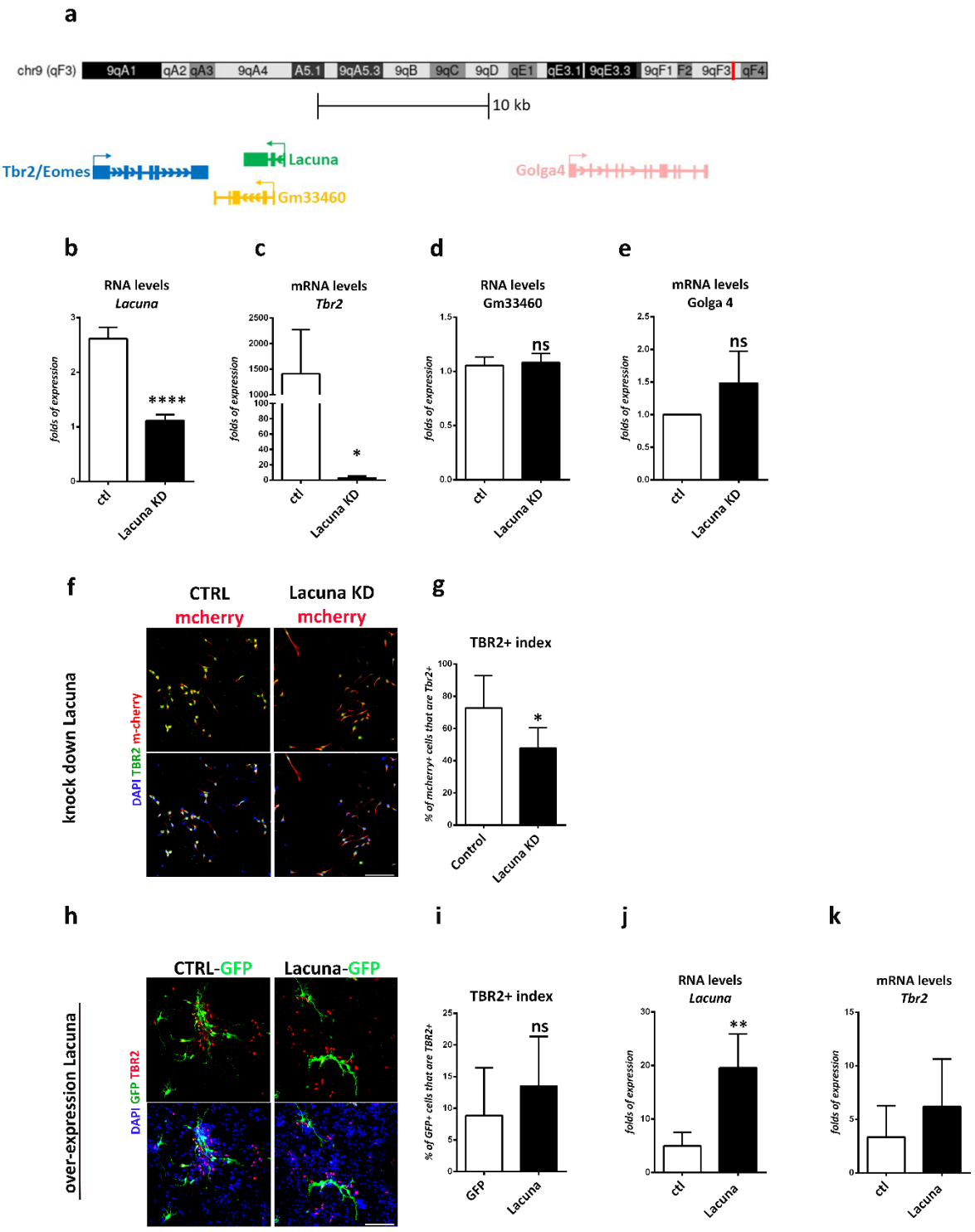

a
chr9 (qF3)
9qA1
qA2
qA3
9qA4
A5.1
9qA5.3
9qB
9qC
9qD
qE1
qE3.1
9qE3.3
9qF1
F2
9qF3
qF4
10 kb
Tbr2/Eomes
Lacuna
Gm33460
Golga4
b
RNA levels
Lacuna
folds of expression
****
ctl
Lacuna KD
c
mRNA levels
Tbr2
folds of expression
*
ctl
Lacuna KD
d
RNA levels
Gm33460
folds of expression
ns
ctl
Lacuna KD
e
mRNA levels
Golga 4
folds of expression
ns
ctl
Lacuna KD
f
knock down Lacuna
CTRL
mcherry
Lacuna KD
mcherry
DAPI TBR2 m-cherry
g
TBR2+ index
% of mcherry+ cells that are Tbr2+
*
Control
Lacuna KD
h
over-expression Lacuna
CTRL-GFP
Lacuna-GFP
DAPI GFP TBR2
i
TBR2+ index
% of GFP+ cells that are TBR2+
ns
GFP
Lacuna
j
RNA levels
Lacuna
folds of expression
**
ctl
Lacuna
k
mRNA levels
Tbr2
folds of expression
ctl
Lacuna

Figure 9. *Lacuna* is necessary for *Tbr2/ Eomes* expression in mouse Neural Stem Cells. (a) Scheme of *Lacuna* and *Tbr2/Eomes* locus on mouse chromosome 9. Despite their vicinity, *Gm33460* and *Golga4* are not affected by guide RNAs targeting *Lacuna*. **(b)** RNA levels of lncRNA *Lacuna* upon Lacuna knockdown **(c)** mRNA levels of *Tbr2* upon Lacuna knockdown **(d)** RNA levels of *Gm33460* upon Lacuna knockdown **(e)** mRNA levels of *Golga4* upon Lacuna knockdown **(f)** Mouse Neural Stem cells were transfected with dCas9-KRAB-mcherry and sgRNA1 targeting *Lacuna* (Lacuna KD-mcherry) or no guide RNA (CTRL-mcherry). They were immunostained for TBR2 (green), mcherry (red) and labeled with DAPI. Scale bar: 100 µM **(g)** Quantification of TBR2 positive cells in dCas9-KRAB-mcherry positive mouse Neural Stem cells (Control: 72,66 ± 7,624%, Lacuna KD: 47,75 ± 4,825%, $p<0.05$). **(h)** *Lacuna-GFP* and *Control-GFP* transfected mouse Neural Stem cells were immunostained for TBR2 (red), GFP (green) and labeled with DAPI. Scale bar: 100 µM **(i)** Quantification of TBR2 positive cells in transgene positive mouse Neural Stem cells (GFP: 30,43 ± 6,023%, Lacuna: 18,52 ± 3,704%, $p>0.05$). **(j)** RNA levels of lncRNA *Lacuna* upon Lacuna overexpression **(k)** mRNA levels of *Tbr2* upon Lacuna overexpression**.** For all cases, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Lockd lncRNA is expressed in developing mouse brain and neighbors *Cdkn1b* gene

In the second part of my thesis, we focused on a pair of lncRNA/protein coding gene with an opposite expression pattern. Thus, we decided to study the lncRNA *RP23-45G16.5*, also named *Lockd*. This lncRNA is transcribed by a genomic locus 4 kb 3' to the *Cdkn1b* gene, which encodes for $p27^{Kip1}$ protein, a Kip1 cyclin dependent kinase inhibitor. *Lockd* is a 434 nt polyadenylated lncRNA containing two exons. Considering the key role of $p27^{Kip1}$ protein in cellular proliferation, differentiation, migration and oncogenesis in the central nervous system, we sought to investigate the functional correlation between these two genes/transcripts of the same genomic locus. Previous studies have reported that *Lockd* positively regulates *Cdkn1b* through an enhancer-like cis element in an erythroid cell line [127], yet our preliminary expression data from RNA-seq analyses, previously performed in our group, indicated that these transcripts exhibit opposite expression patterns.

To further study the correlation between these two genes, we started by analyzing the detailed expression pattern of *Lockd* during neural development. In particular, we first confirmed that *Lockd* is highly expressed in the developing mouse telencephalon. Its expression is detected in early developmental stages (E10), peaks at E12 and then it is gradually declining until P0 (Figure 10). In this case, *Lockd* expression during CNS development shows an opposite pattern than its neighboring *Cdkn1b* gene, which is first detected in E10 embryonic telencephalon, is gradually upregulated and peaks at E18 (Figure 10). This expression pattern is consistent with the role of p27 in suppressing proliferation and promoting differentiation and migration.

We also studied the endogenous *Lockd* expression in primary NSCs and specifically in the two conditions used to culture them. Interestingly, *Lockd* expression is higher in plus growth factors (+GF) condition, where NSCs proliferate and self-renew, in comparison to minus growth factors (-GF) condition, where NSCs start to differentiate (Figure 12e). This was a strong indication that *Lockd* transcript is involved in the regulation of proliferation.

Figure 10

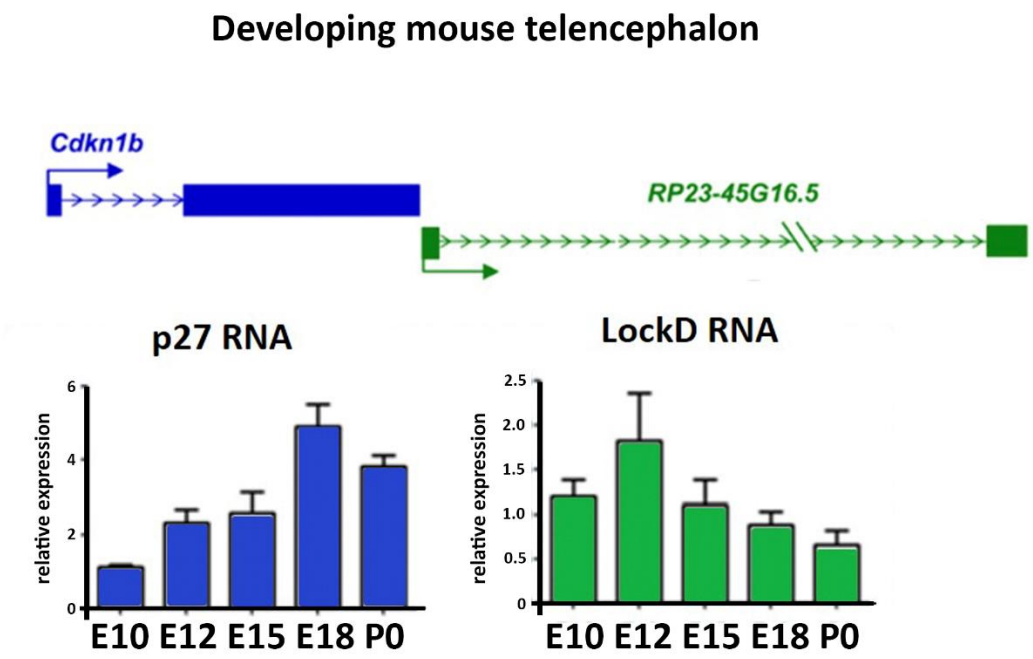


Figure 10. Expression profiles of *Cdkn1b* and *LockD* during mouse brain development. The corresponding genetic locus is shown schematically. RNA samples were collected from mouse embryonic telencephalon in each developmental stage, as indicated.

Lockd overexpression increases proliferation in N2A cells

Similarly to our previously presented studies (Lacuna), a mixture of two plasmids (pCAGGs-Lockd and pCAGGs-GFP or pCAGGS empty and pCAGGS-GFP) was used to transfect N2A cells. Upon *Lockd* overexpression, N2A cells exhibited increased proliferation as shown by BrdU incorporation assays (Figure 11a-b). Interestingly, *Lockd* overexpression results in a significant decrease of *Cdkn1b* (encoding for $p27^{Kip1}$) expression as shown by real-time RT-qPCR (Figure 11c-d). This effect on *Cdkn1b* could possibly explain the increase in proliferation rate upon *Lockd* overexpression, as $p27^{Kip1}$ promotes cell cycle exit.

Figure 11

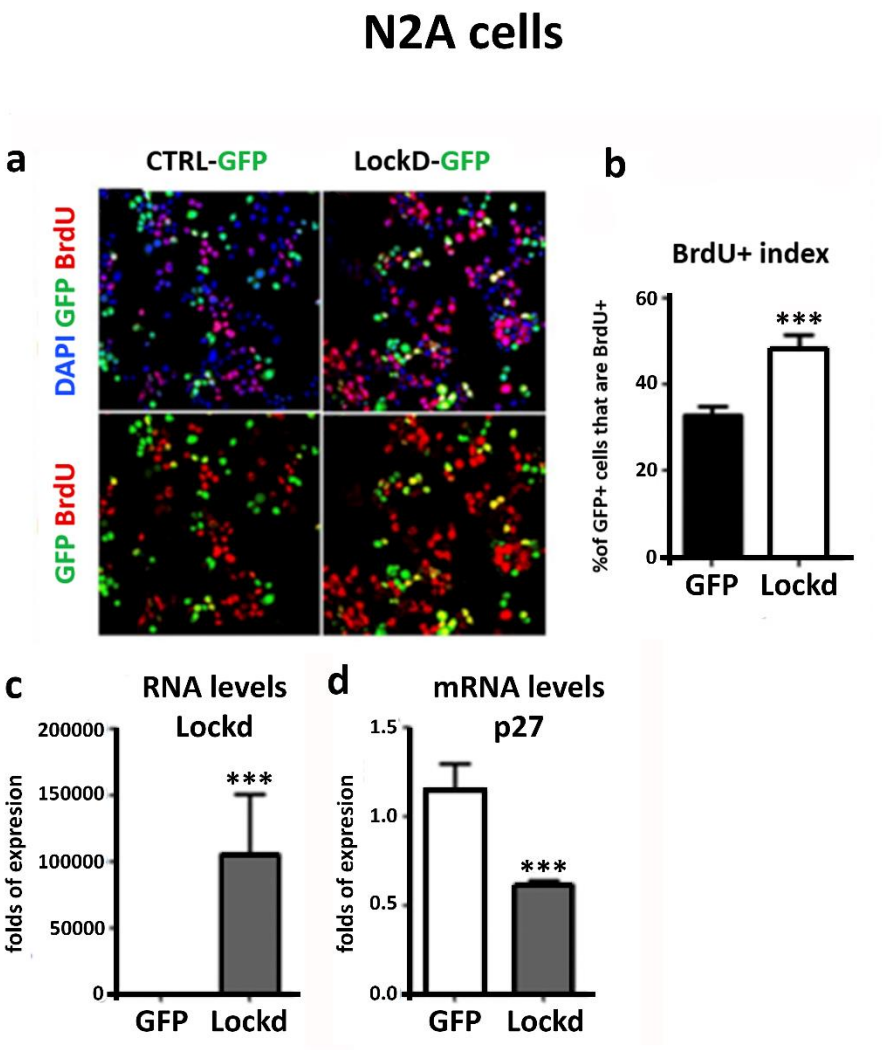


Figure 11. *Lockd* overexpression increases proliferation in N2A cells. N2A cells were transfected with *pCAGGs-Lockd* and *pCAGGs-GFP* plasmids (Lockd-GFP) or pCAGGs empty and pCAGGs-GFP plasmids (CTRL-GFP) **(a)** They were treated with BrdU for 2h and then fixed and stained with anti-GFP (green), anti-BrdU antibody (red) and 4, 6-diamidino-2-phenylindole (DAPI) **(b)** Quantification of BrdU incorporation in GFP positive N2A cells (GFP: 34,29 ± 1,71%, Lockd: 46,29 ± 3,67%, p<0.001) **(c)** RNA levels of lncRNA *Lockd* upon *Lockd* overexpression in N2A cells **(d)** mRNA levels of p27 upon *Lockd* overexpression in N2A cells. For all cases, * p<0.05, ** p<0.01, *** p<0.001

Lockd overexpression increases proliferation in NSCs

Then, we wanted to investigate the involvement of *Lockd* in proliferation in a more physiological and developmental context. To this end, the same mixture of plasmids was used to overexpress *Lockd* in primary NSCs derived from E14 mouse embryonic cortices. Again, upon *Lockd* overexpression, proliferation was increased in NSCs cultures in the presence of growth factors as shown by BrdU incorporation assays (Figure 12a-b). Similarly to the experiments performed in N2A cells, *Lockd* overexpression in NSCs results in a significant downregulation of *Cdkn1b* (encoding for $p27^{Kip1}$), as shown by real-time RT-qPCR (Figure 12c-d). This is also in accordance with the downregulation of *Lockd* when NSCs are cultured without growth factors, meaning in conditions that facilitate differentiation.

Figure 12

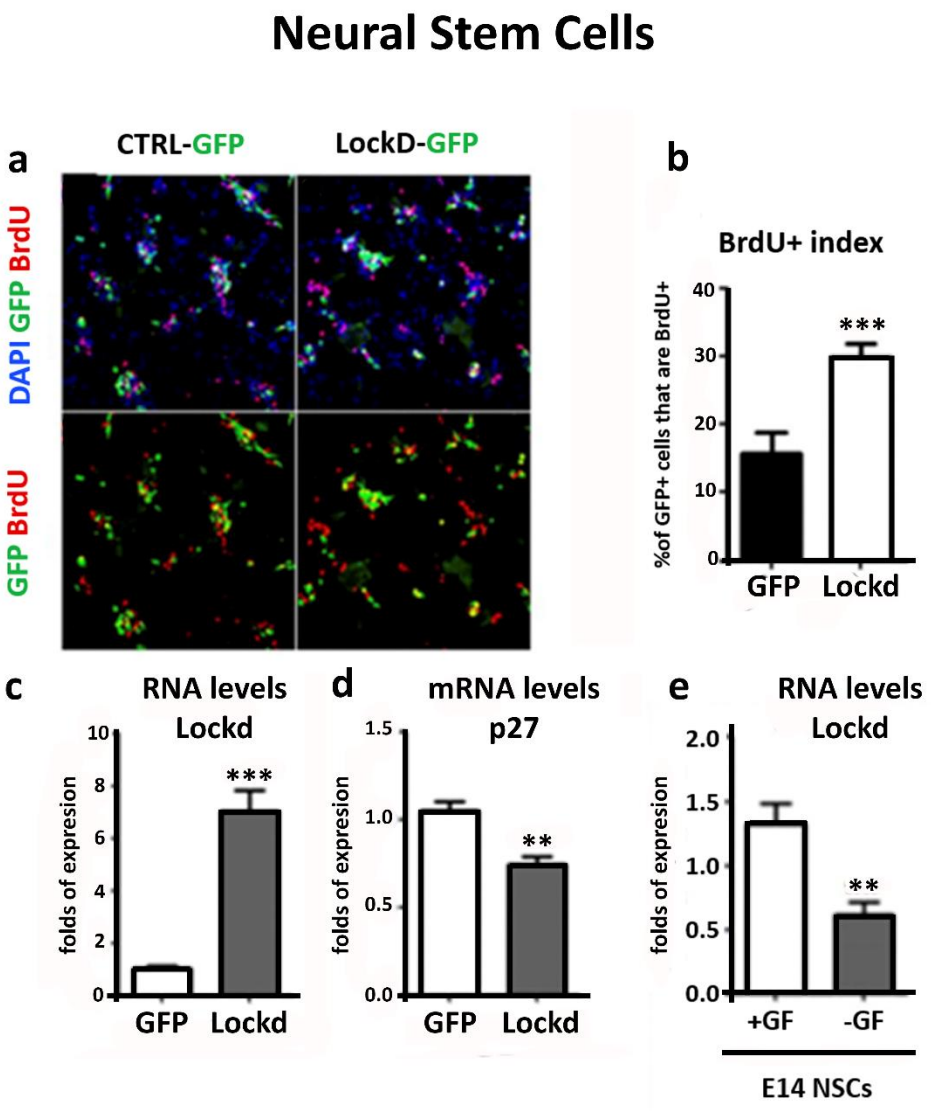


Figure 12. *Lockd* overexpression increases proliferation in mouse Neural Stem Cells. Mouse Neural Stem Cells were transfected with *pCAGGs-Lockd* and *pCAGGs-GFP* plasmids (Lockd-GFP) or pCAGGs empty and pCAGGs-GFP plasmids (CTRL-GFP) **(a)** They were treated with BrdU for 2h and then fixed and stained with anti-GFP (green), anti-BrdU antibody (red) and 4, 6-diamidino-2-phenylindole (DAPI) **(b)** Quantification of BrdU incorporation in GFP positive Neural Stem Cells (GFP: 15,52 ± 2,51%, Lockd: 29,25 ± 1,79%, $p<0.001$) **(c)** RNA levels of lncRNA *Lockd* upon Lockd overexpression in Neural Stem Cells **(d)** mRNA levels of p27 upon *Lockd* overexpression in Neural Stem Cells. **(e)** RNA levels of *Lockd* in Neural Stem Cells from E14 mouse embryonic brain cultured in two conditions: with and without growth factors. For all cases, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Discussion

More than 70% of the human genome is transcribed, but much less is finally translated into proteins. Protein-coding genes represent approximately the 34% of all human annotated genes. All the rest are non-coding genes and pseudogenes. Among them, the class of the long non-coding RNAs represent the 27% of human annotated genes. LncRNAs are a heterogeneous class of RNA transcripts of at least 200 nucleotides in length that lack an evident open reading frame. They are transcribed from various genomic loci and act through a wide range of mechanisms. They combine uniquely three specific features: a) their intrinsic nucleic acid nature that allows them to interact with proteins and other nucleic acids, b) their three-dimensional structures, c) their length that it is enough to include multiple functional domains. This combination renders lncRNAs as spectacularly diverse molecules, a feature that is very valuable in processes that require spatiotemporal coordination of many factors, such as neurodevelopment.

Intriguingly, the lncRNAs that are expressed in the brain are preferentially harbored by genomic loci in vicinity of brain-specific, transcriptionally active during development, protein coding genes. Our preliminary data confirmed this piece of knowledge, and we went one step further by generating a rich list of lncRNA – Transcription Factor pairs, each one consisting of one lncRNA expressed in the brain during development and a neighboring protein coding gene encoding for a crucial transcription factor. This list (Table 1) contains information on putative lncRNA – Transcription factor gene that may form the basis for many interesting hypotheses on the molecular and functional interplays between these transcripts that may also function on diverse developmental stages of embryonic brain.

This list was the primary source for our study. We selected six pairs to investigate further. One of our first observations was that the expression profile of some lncRNAs often followed the same pattern of the neighboring transcription factor gene (Figure 1c-f). A distinctive exemption of this motive was *Ariel* and *Prox1*, where their expression patterns were actually opposite. Further investigation of *Ariel* showed that this lncRNA is necessary and sufficient for proper differentiation of NSCs and that it antagonizes *Prox1* (manuscript under preparation).

Our experimental pipeline led us to focus our efforts on *TCONS_00034309*, named by us as *Lacuna*, as it is not annotated yet and it is the first time it is studied in any system or tissue. *Lacuna* is located on the chromosome 9 of mouse genome approximately 1.5 kb away from *Tbr2 / Eomes* gene. It is 1661 nucleotides long and it consists of three exons (Figure 4). *Lacuna* expression starts around E14.5 in the ventricular zone of the developing cortex, but later, when its expression peaks on E16.5, *Lacuna* is

expressed in the ventricular zone and the cortical plate. On the other hand, *Tbr2 / Eomes* gene is expressed gradually until E16 and then it is abruptly downregulated (Figure 3) [22,128-130]. This similarity in their expression patterns suggests a common regulation of these two genes or a synergistic interaction between them. Indeed, with our knockdown strategy in primary NSCs cultures, we showed that *Lacuna* is necessary for *Tbr2 / Eomes* expression. However, exogenous overexpression of *Lacuna* is not sufficient to upregulate or in any way affect *Tbr2 / Eomes* expression in NSCs (Figure 9). This difference seemed at first paradoxical, yet it probably indicates that *Lacuna* transcript can regulate *Tbr2/Eomes* expression only *in cis*. To confirm this in the future, it would be interesting to examine whether *in cis* activation of *Lacuna* expression could also lead to the transcriptional induction of *Tbr2* gene. For this purpose, we could use a specific activation system such as CRISPR-VPR to target the endogenous *Lacuna* and activate its expression.

The downregulation of *Tbr2 / Eomes* upon Lacuna knockdown revealed that *Lacuna* transcript - and not *Lacuna* DNA element- is involved in *Tbr2 / Eomes* regulation. The strategy we used (dCas9-KRAB effector system) leaves the DNA of the locus intact and suppresses transcription by specifically reducing the RNA transcript of choice. The principle of this system is that KRAB protein, which is fused with the dCas9, directly interacts with a number of chromatin modulators able to generate a genomic microenvironment resembling a “closed” chromatin structure (repressive for transcription). Thus, recruitment of KRAB and associating factors to the gene of interest renders it inactive without changing the underlying DNA sequence [124,125,131]. Following this experimental strategy, we confirmed that this system is sufficient to downregulate *Lacuna* gene expression and at the same time to also suppress *Tbr2* gene expression. A key technical question arising from these observations is whether the concomitant effect on *Tbr2* is non-specific (artifact) due to the recruitment of chromatin modulators in the *Lacuna* genomic locus. Search in the published literature suggested that this system is not affecting other genes in long distance, favoring a specific effect on *Tbr2*. To further exclude this possibility, we also examined whether other genes in the *Lacuna* genomic region are also affected by the recruitment of dCas9/KRAB system on *Lacuna* gene. Therefore, we were able to show that two other genes that are located in close proximity to *Lacuna* and *Tbr2* are not affected, so we conclude that *Tbr2 / Eomes* downregulation is specific and due to *Lacuna* knockdown (Figure 9). Thus, our hypothesis is that *Lacuna* acts *in cis* facilitating the expression of *Tbr2 / Eomes* gene in the appropriate developmental time window and in the appropriate zone of the developing mouse cortex. It would also be interesting to investigate the direct interaction between *Lacuna* transcript and regulatory DNA sequences of *Tbr2* gene locus by ChIRP (Chromatin Isolation by RNA Purification).

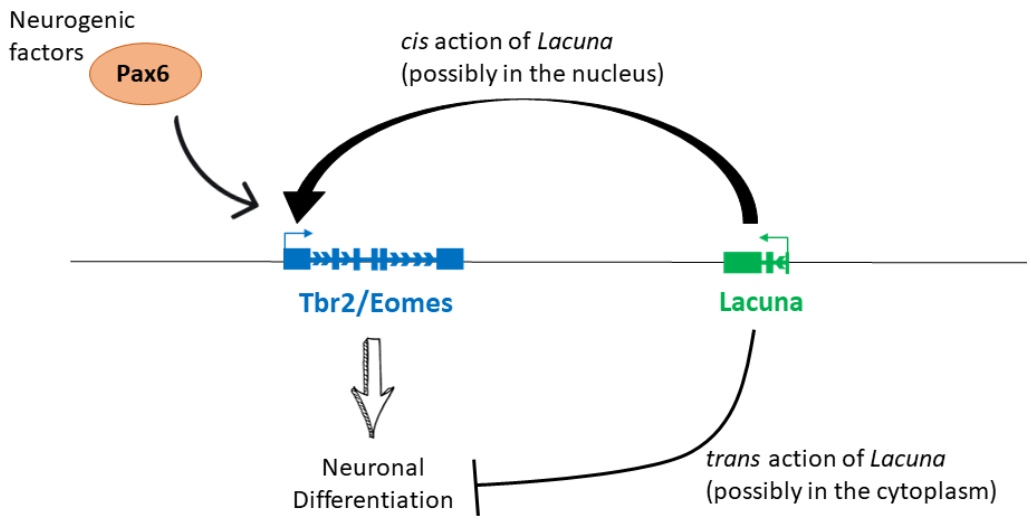
The *in cis* action of *Lacuna* is also supported by the presence of *Lacuna* transcripts in the nucleus of NSCs (Figure 3), indicating a function related with the regulation of gene expression. However, *Lacuna* is found to be equally distributed between the nucleus and cytosol suggesting that it has also additional roles that we may need to carefully consider in our study. In order to further investigate this lncRNA, we continued with gain-of-function and loss-of-function experiments in primary NSCs cultures to detect any possible effects on NSC properties, such as proliferation and differentiation. Indeed, one of the most important findings is that *Lacuna* affects neurogenesis. Overexpression of *Lacuna* negatively affects the population of post-mitotic neurons (Figure 6) and conversely, *Lacuna* knockdown promotes the differentiation of NSCs into neurons (Figure 8). Therefore, we believe that *Lacuna* is also involved in the molecular mechanisms that inhibit neuronal differentiation.

A rather surprising finding in our study is the effect of *Lacuna* on *Olig2* transcription factor and more specifically, on the Olig2+ population of differentiating NSCs. It has been previously reported that *Olig2* misexpression in neural stem cells elicits neurogenesis defects [132] and that *Olig2* has also anti-neuronal functions except of its well-known pro-neural functions in different developmental stages and depending on its phosphorylation state [133]. It is known that *Olig2* antagonizes *Ngn2* and inhibits the premature expression of post-mitotic motor neuron genes holding progenitor cells in reserve for later differentiation [134]. In our study, neurogenesis defects in *Lacuna* overexpressing NSCs are accompanied by an increase in the Olig2+ cells suggesting that these NSCs are not able to differentiate properly into neurons but are kept in an undifferentiated state. Indeed, proliferation rate is not affected, whereas Nestin+ cells are increased supporting the notion that these cells are preserving their stemness. The exact opposite effects were observed in NSCs that were lacking *Lacuna*. In our loss-of-function experiments, *Lacuna* knockdown resulted in increased numbers of post-mitotic neurons, less Olig2+ cells and reduced Nestin+ cells. Taken together, these observations indicate that *Lacuna* RNA is involved in the regulation of neurogenesis in NSCs, probably through an Olig2-mediated pathway. It would be of great interest to dissect in more details this involvement, e.g., possible effects on the phosphorylation of Olig2, other markers like Pax6 and Tbr1 or data derived by RNA-seq analysis on *Lacuna* gain- and loss-of-function experiments.

The next intriguing question was whether the effects of *Lacuna* on neuronal differentiation are dependent on the *in cis* action of this lncRNA on the *Tbr2* transcription factor. It seems that the involvement of *Lacuna* in the neuronal differentiation of NSCs is independent of its *in cis* action on *Tbr2*. This notion is supported by two main facts: First, it is reported in many different contexts that *Tbr2*

promotes neuronal differentiation [22,25,26] and it is essential for intermediate neural progenitor specification [135]. There are even reported patients suffering from microcephaly, polymicrogyria and corpus callosum agenesis that lack *Tbr2* transcript due to a chromosomal translocation [27]. These data show that *Tbr2* exerts an opposite function than what we observe in our experiments. Second, *Lacuna* effects on neuronal differentiation are not only shown in *Lacuna* knockdown where *Tbr2* is downregulated, but they are also evident in our gain-of-function experiments, where *Tbr2* expression remains unaffected.

Thus, we propose a hypothetical model (shown below) where *Lacuna* exerts a *Tbr2*-independent inhibitory effect on neuronal differentiation of NSCs via a mechanistic action in the nucleus and/or in the cytoplasm. Lacuna is also able to facilitate *Tbr2* expression *in cis*. Since Tbr2 promotes neuronal differentiation, it is extremely fascinating to understand why the same molecule exerts two seemingly opposite actions. Toward this direction, we would like to hypothesize that *Lacuna* exerts both actions in an effort to finetune and link into a cross-regulatory loop these two molecular pathways (a) inhibition of differentiation and (b) induction of *Tbr2* expression. This finetuning action may contribute to the regulatory networks that control the developmental process in a short and precise time window and/or in a very specific cell population. A finding that could support this hypothesis is the dual localization of *Lacuna* transcript not only in the subcellular level (cytoplasm / nucleus) but also in the different zones of the mouse developing brain (cortical plate / ventricular zone) (Figure 3). The putative dual role of *Lacuna* could hopefully be identified through in vivo experiments, where developmental events are observed in greater detail, and it may unravel valuable information about the undiscovered events of brain development.



Hypothetical model of Lacuna action

In the second part of this thesis, we focused on a different pair of lncRNA – protein coding gene; this time not involving a transcription factor, but a cyclin dependent kinase inhibitor, p27. This factor has an established role in proliferation as it promotes cell cycle exit, but also it retains an essential part in the differentiation and migration in the central nervous system [33,35,36,136]. *Lockd* is the neighboring lncRNA and it was already studied in an erythroid cell line, where it is reported that it positively regulates p27 expression through its associated enhancer-like cis element. In that study, *Lockd* transcript is described as dispensable for the regulation of the gene encoding for p27 (*Cdkn1b*) [127].

In our study, we first showed that *Lockd* is expressed in the mouse developing brain in an opposite expression pattern than p27 (Figure 10). Our first question, emerging from the involvement of p27 in the cell cycle exit events, was whether *Lockd* affects proliferation of N2A neuroblastoma cell line. Indeed, *Lockd* overexpression increases proliferation of N2A cells and interestingly, it negatively affects the expression of *Cdkn1b* gene (Figure 11). Taken together, our data support a totally different function of *Lockd* between nervous system and erythroid progenitor cell, as proposed by Paralkar et al. In our case, the effect of *Lockd* on *Cdkn1b* expression is the opposite and also, *Lockd* transcript is sufficient to modulate the p27 gene regulation when we exogenously overexpress it. Moreover, as this finding was fascinating and our scientific interest focuses on neurodevelopment, we copied this experimental design to neural stem cells. Initially, we investigated the endogenous *Lockd* expression in NSCs and we showed that it is expressed highly when the NSCs are cultured in the presence of growth factors, where they primarily proliferate. Interestingly, when the NSCs are transferred to media without growth factors, a condition that facilitates their differentiation and inhibition of proliferation, *Lockd* is significantly

downregulated. This is in accordance with our findings from *Lockd* overexpression in NSCs, where, similarly to our experiments in N2A cells, *Lockd*-overexpressing NSCs proliferate at much higher rates than the controls. Additionally, p27 is once again downregulated upon overexpression of *Lockd* (Figure 12).

Taken together, we showed that *Lockd* transcript negatively regulates the expression of p27 in neuroblastoma cells and in NSCs with subsequent positive effects on proliferation properties. The fact that this interplay was identified in two different cell type cultures makes our findings really intriguing, as p27 is involved in both developmental processes and tumorigenesis. This study is still at its beginning with many possible extensions in the field of developmental biology and cancer biology. Considering that p27 is a tumor suppressor gene for nervous system tumors and other cancer types, it would be extremely interesting to examine whether *Lockd* can function as an oncogene in these tissues.

In conclusion, in this doctoral thesis, we studied the role of lncRNAs in mammalian brain development with the aim to contribute to the knowledge in the fast-evolving fields of lncRNA biology and neurodevelopmental biology. From the RNA World Hypothesis to the messenger RNA model of the central dogma of molecular biology in the 1950s, and from the first discovery of non-coding RNAs in bacteria to the first identified lncRNAs in the pre-genomic era, RNA molecules are being extensively studied. Of note, in 2021 -year of publication of this thesis- RNA vaccines are thrown into the battle of Covid19 pandemic. LncRNAs, the most recently identified class of RNA molecules, are a rapidly emerging field that could elucidate key developmental processes but could also serve as therapeutic targets.

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Curriculum Vitae

EDUCATION

- 05/2014 – 06/2021: PhD entitled “The role of lncRNAs in the development of mammalian brain”, National Kapodistrian University of Athens, School of Science, Faculty of Biology

- 09/2011 – 07/2013: MSc “Molecular Medicine”, National Kapodistrian University of Athens, Medical School

- 2006 – 2011: BSc “Biology”, National Kapodistrian University of Athens, School of Science, Faculty of Biology

PUBLICATIONS

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TRAINING

• 05/2014 – 06/2021: PhD in Biomedical Research Foundation of Academy of Athens supervised by Dr. P. Politis and co-supervised by Dr G. Diallinas and Dr. S. Efthimiopoulos, University of Athens, entitled "The role of lncRNAs in the development of mammalian brain"

• 03/2012 – 05/2014: Rotation and master thesis under the MSc "Molecular Medicine", in the Developmental Biology Laboratory, B.R.F.A.A., supervised by Dr. A. Gavalas entitled "Long-term effects of aldehyde dehydrogenase 1b1 in pancreatic islet functionality"

• 10/2011 – 03/2012: Rotation under the MSc "Molecular Medicine", in the Genetics Laboratory, B.R.F.A.A., supervised by Dr. C. Zervas entitled "The relation between parvin and ILK in the developing epithelia of Drosophila melanogaster"

• 10/2010 – 04/2011: Training under the Erasmus Placement Program in the Biotechnology Laboratory, IATA, CSIC, Valencia, supervised by Dr. M. Orejas & Dr. A. MacCabe entitled "Molecular characterization of genes involved in the production of fungal hydrolases"

• 07 - 08/2009: Voluntary work at the Microbiology Faculty of the Health Care Center, Milos Island, Greece

FELLOWSHIPS AND AWARDS

• 03/2020: Fulbright scholarship "Greek Visiting Research Students" to visit Dr. D. Lim's lab in UCSF, San Francisco (July 2021 – November 2021)

• 07/2019: Travel grant award from Federation of European Neuroscience Societies to attend FENS Regional Meeting in Belgrade, Serbia

• 05/2017 – 11/2018: Scholarship funded by the Act "Scholarships grant program for the second round of postgraduate studies" from resources of OP "Human Resources Development, Education and Lifelong Learning" 2014-2020, co-funded by the European Social Fund (ESF) and the Greek State

• 2006: Honorary award from the Hellenic Ministry of Education for entering Faculty of Biology, School of Science, National and Kapodistrian University of Athens among the first twenty students (4th place) after Panhellenic examinations

CONFERENCE PRESENTATIONS

• 20/2020: “Long non-coding RNA – Transcription factor regulatory networks in mammalian neural stem cells”, Interactive talk, Neuromatch 3.0 online

• 10/2019: Talk entitled “Long non-coding RNA – Transcription factor regulatory networks in mammalian brain development”, 28th Meeting of the Hellenic Society for Neuroscience, Heraklion, Greece

• 07/2019: Talk entitled “Long non-coding RNA – Transcription factor regulatory networks in mammalian brain development”, Blitz sessions - FENS Regional Meeting in Belgrade, Serbia

• 07/2019: Poster presentation entitled “Long non-coding RNA – Transcription factor regulatory networks in mammalian brain development”, FENS Regional Meeting in Belgrade, Serbia

• 12/2017: Poster presentation entitled “The role of lncRNAs in the development of mammalian brain”, 27th Hellenic Society for Neuroscience Meeting, Athens

• 11/2017: Poster presentation entitled “The role of lncRNAs in the development of mammalian brain”, 68th HSBMB Conference, Evgenidio Inst, Athens

• 12/2013: Talk entitled: «Aldh1b1 regulates the timing in pancreas lineage segregation and beta cell maturation», 64th HSBMB Conference, Evgenidio Inst, Athens (speaker)

• 11/2013: Talk entitled: «Aldh1b1 regulates the timing in pancreas lineage segregation and beta cell maturation», 1st Annual Conference of the German Stem Cell Network, Berlin (speaker)

• 04/2013: Talk entitled: «ALDH activity in pancreas development and regeneration», 11th German Islet Workshop, CRTD, Dresden (speaker)

• 01/2004: Presentation of suggestions entitled: “The commercialization of knowledge, technology and media”, part of the 35th annual session of the Hague International Model United Nations, sponsored by the United Nations (contributor)

TEACHING EXPRERIENCE

• 10/2019 – 07/2020: Coordinator of module “Human Physiology” in BSc (Hons) “Podiatry”, School of Health Sciences, Metropolitan College, Athens

• 09/2016 – 01/2018: Private lessons to high school and undergraduate students (Biology, Chemistry)

• 09/2014 – 05/2015: Volunteer in W.R.O. HELLAS (World Robot Olympiad) teaching science to primary school students (STEM education)

LANGUAGES & COMPUTER SKILLS

English Proficiency (University of Cambridge)

French C1 (D.A.L.F.)

Spanish B2 (Intermedio)

«Cambridge Skills Award in Information Technology»

MEMBERSHIPS

Member of FENS (Federation of European Neuroscience Societies)

Member of HSfN (Hellenic Society for Neuroscience)

Member of PUB (Panhellenic Union of Bioscientists)

Regular member of “University education and Research Committee” of Panhellenic Union of Bioscientists

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REVIEW ARTICLE

Targeting Long Non-Coding RNAs in Nervous System Cancers: New Insights in Prognosis, Diagnosis and Therapy

Nikos Malissovas[#], Elpinickie Ninou[#], Artemis Michail[#] and Panagiotis K. Politis[*]

Center for Basic Research, Biomedical Research Foundation of the Academy of Athens, 4 Soranou Efesiou Str, 115 27, Athens, Greece

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Abstract: Long non-coding RNAs (lncRNAs) constitute one of the most broad and diverse classes of cellular transcripts, playing key roles as regulatory molecules in many biological processes. Although the biology of lncRNAs is a new and emerging field of research, several studies have already shown that alterations in the expression of lncRNAs are associated with the development and progression of cancer in different organs and tissues, including central and peripheral nervous system. In this review, we summarize the oncogenic and tumor suppressive roles of lncRNAs in malignant tumors of the nervous system, such as glioma and neuroblastoma, focusing on their functional interactions with DNA, other RNA and protein molecules. We further discuss the potential use of lncRNAs as biomarkers for diagnosis, prognosis and tumor treatment. Gaining insight into the functional association between nervous system malignancies and lncRNAs could offer new perspectives to the development of promising therapeutic tools against cancer.

Keywords: Glioblastoma, neuroblastoma, HOTAIR, XIST, MALAT1, MEG3, I-BET, circulating lncRNAs.

1. INTRODUCTION

Interrogation of the non-coding genome has revealed numerous key players with critical functions in cancer biology. Accordingly, a continuously expanding list of previously unidentified long non-coding RNAs (lncRNAs) has been recently characterized with regulatory functions in the development of multiple human diseases, including cancer [1-4]. Functional studies have shown that several of these lncRNAs could regulate gene expression at transcriptional, post-transcriptional and epigenetic levels [5]. In addition, differential expression of many lncRNAs has been associated with cancer initiation and progression as well as cellular and molecular mechanisms associated with tumorigenesis [3, 6].

In the context of nervous system, lncRNAs have become the frontline of cancer research. Multiple studies are focusing on the implication of lncRNAs and their underlying signaling pathways in the initiation and progression of the two major types of nervous system-related cancers: gliomas [7] and neuroblastomas [8]. Gliomas are the most common primary tumors in the brain [9] and they are classified as astrocytomas, oligodendrogliomas, ependymomas or mixed tumors (oligoastrocytomas), with grades of I to IV according to World Health Organization (WHO). Glioblastoma multiforme (GBM) is the most aggressive tumor of the central nervous system and represents 15% of all brain tumors [10]. There has been an extreme advance in the treatment of GBM with a combination of chemotherapy - typically temozolomide - and radiation following surgical resection [11, 12]. Without treatment, survival is around three months. The average survival time is extended to 12 – 15 months, but still the prognosis remains poor and the cancer usually recurs [10]. Regarding Neuroblastoma, it has an early onset during childhood that emerges from the developing autonomic nervous system [13-16]. There is a great variability and heterogeneity in the clinical symptoms. Although spontaneous regression is observed in a subgroup of neuro-

*Address correspondence to Panagiotis K. Politis at the Center for Basic Research, Biomedical Research Foundation of the Academy of Athens, 4 Soranou Efesiou Str., 115 27, Athens, Greece;
Tel/Fax: ++30-210-6597479, ++30-210-6597545;
E-mail: ppolitis@bioacademy.gr
[#]*These authors contributed equally to this work*

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blastoma cases, some other tumors progress to highly aggressive metastatic disease with a poor overall survival rate. Less than 40% of children with high-risk neuroblastoma are expected to achieve long-term cure, in spite of the dramatic escalations in the intensity of provided therapy [13, 17]. Collectively, these clinical findings underline the necessity for further investigation into the mechanisms of malignant transformations in the nervous system. Thus, the recent involvement of lncRNAs in these malignancies has generated new hopes for understanding, stratifying and combating nervous system tumors.

To this direction and similarly to other human cancers, it has been described that many lncRNAs exhibit deregulated expression upon nervous system tumors pathogenesis [7, 18-19]. It has also been suggested that lncRNAs could regulate various oncogenic processes such as cellular proliferation and apoptosis, stem cell differentiation, cell motility and tumor metastasis [20, 21]. LncRNAs differential expression reflects clinical phenotypes and patient prognosis. In addition, lncRNAs may give a unique opportunity to be exploited as potential therapeutic targets [22]. Therefore, in this review, we first provide an overview of the genome-wide screening studies that identified dysregulated expression patterns of lncRNAs during tumor initiation and progression, then, summarize the role of lncRNAs in the pathophysiology of nervous system-related cancers and the potential underlying mechanisms. Finally, we discuss the potential use of lncRNAs as therapeutic targets and/or biomarkers for cancer diagnosis and prognosis.

2. DIFFERENTIAL EXPRESSION OF LNCRNAS IS ASSOCIATED WITH TUMOR INITIATION AND PROGRESSION IN NERVOUS SYSTEM MALIGNANCIES

Recent efforts to profile the transcriptome in a genome-wide manner indicate that lncRNAs may be key regulators in nervous system cancers, demonstrating differential expression patterns in tumor versus healthy tissues along with different cancer types and malignancy grades [23-25]. Differential expression of lncRNAs in tumors suggests a potential role of lncRNAs in the initiation, progression and prognosis of these diseases.

Regarding cancer initiation and progression, the lncRNA expression profiles are significantly altered in gliomas when compared with normal brain tissue. Han *et al.,* (2012) analyzed and identified 1308 deregulated lncRNAs (654 up-regulated and 654 down-regulated), that had differential expression in GBM and normal samples. Among them, ALSNC22381 and ALSNC20819 are particularly interesting since they could target IGF-1, which plays a key role in glioma recurrence [25, 27]. Similarly, two independent studies revealed elevated levels of CRNDE and HOTAIRM1 expression in gliomas [28, 29]. In another study, Grzmil *et al.* (2011) identified 213 differentially expressed lncRNAs in 5 GBM cell lines (LN215, BS149, LN319, LN229 and LN018) when compared with normal astrocytes, and 147 lncRNAs in 30 glioma samples (8 astrocytomas, 7 oligodendrogliomas, 12 primary GBM and 3 secondary GBM) when compared with normal brain tissue. In addition, Murat *et al*. (2008) found a set of 81 differentially expressed lncRNAs by comparing 80 glioblastoma samples collected from human patients. 37 of these lncRNAs were up-regulated and 44 were down-regulated in these tumors. Finally, recent reports indicate that the expression of MALAT1, POU3F3 and H19 is associated with more aggressive glioma phenotype [30-32]. These findings suggest that lncRNAs play a crucial role in the initiation of brain tumorigenesis, since deregulation of their expression could be an early event in cancer biology.

Several studies in recent years have also indicated the involvement of lncRNAs in glioma progression. For instance, by comparing low-grade and high-grade astrocytic tumors Zhang *et al.* (2012) found a set of 12 lncRNAs strongly linked to the progression of astrocytoma [21]. In particular, HOTAIRM1 and CRNDE were found highly up-regulated, while MEG was down-regulated. Moreover, H19 expression levels were positively associated with increased tumor malignancy grades, while both RFPL1S and PAR5 were negatively associated, suggesting a potential significant role for lncRNAs in glioma progression [33].

Moreover, differential expression of multiple lncRNAs has been reported in other nervous system cancers, including neuroblastoma. For example, Scaruffi *et al.* proposed a prognostic prediction model based on the evaluation of the expression data from 481 Transcribed Ultra-Conserved regions (T-UCRs), obtained from 34 high-risk, aggressive neuroblastoma patients using qRT-PCR method [34]. T-UCRs are a subset of highly conserved lncRNAs among mammals. This analysis specifically showed that 28 T-UCRs out of 481 were significantly implicated in patient prognosis and accurately predicted clinical phenotypes. Interestingly, 15 up-regulated T-UCRs were able to discriminate long and short-term survivors. Furthermore, two other parallel studies have analyzed the expression

pattern of these 481 T-UCRs in neuroblastoma tumors [35, 36]. Mestdagh *et al.,* showed from a qRT-PCR analysis in a group of 49 neuroblastoma tumors that 7 T-UCRs are up-regulated in tumors with MYCN amplification. In addition, uc.460, uc.279 and uc.364 T-UCRs display a positive correlation with MYCN gene expression in a larger patient dataset (366 samples). Finally, by using a RNA-seq approach, in a recent study, the transcriptomes of 15 tumors from low and high-risk subtypes of neuroblastoma were analyzed [37]. These data revealed many annotated lncRNAs, including Neuroblastoma Associated Transcript 1 (NBAT1), as differentially expressed in high- and low-risk neuroblastoma types. Analysis of expression profile for NBAT1 in two independent groups (498 and 93) of neuroblastoma tumors revealed that low expression of NBAT1 in patients is correlated to poor clinical outcome.

In addition, many studies identified lncRNAs as prognostic factors. MGC21881, PART1, MIAT, PAR5 and GAS5 were associated with prolonged survival, while KIAA0495 was correlated with poor survival [38]. Other studies showed that MALAT1 was up-regulated in glioma tissues in comparison to adjacent normal brain tissue. MALAT1 up-regulation was associated with poorer overall survival in glioma patients [39]. Finally, another potential prognostic marker that was identified with high expression levels was HOTAIR [40].

3. FUNCTIONAL ANALYSIS OF WELL-CHARACTERIZED LNCRNAS IN GLIOMAS

Numerous lncRNAs have been functionally implicated in the initiation, progression and recurrence of brain tumors. For a number of these lncRNAs, their regulatory roles in critical biological or clinical characteristics of gliomas have been clearly established.

3.1. The Functional Role of HOTAIR (HOX Transcript Antisense Intergenic RNA)

HOTAIR is a key prognostic factor in different cancer types including GBM. HOTAIR has been positively linked to poor prognosis, tumor staging and the molecular subtype of glioma [41]. In addition to lncRNA profiling, Pastori *et al.* by performing single molecule sequencing (SMS) expression analysis found that HOTAIR has extremely elevated expression levels in tumor cells compared to control [26]. In accordance, depletion of HOTAIR transcript in glioma cell significantly reduces their growth [26]. The HOTAIR lncRNA acts as an oncogene in GBM by interacting with epigenetic regulators. The pro-oncogenic activity is mediated through direct binding to PRC2, a chromatin modifying complex, promoting the histone H3K27 trimethylation and consequently leading to epigenetic silencing of downstream effector genes (Fig. **1A**) [42, 43]. In addition, HOTAIR expression is directly regulated by another epigenetic modulator the Bromodomain and extraterminal (BET) domain protein 4 (BRD4). BRD4 is a well-studied protein in GBM and has been shown to directly bind and recruited to HOTAIR, thus, enhancing its expression levels (Fig. **1A**). Moreover, two different groups showed that reduced levels of HOTAIR expression inhibit Glioma Stem Cells (GSCs) differentiation, induce cell cycle arrest and suppress tumor metastasis. These regulatory functions of HOTAIR are explained by its ability to inhibit Wnt/b-catenin/ programmed cell death protein 4 (PDCD4) pathways [44, 45]. Finally, HOTAIR expression in tumor cells can also be activated by c-Myc (Fig. **1A**). There is a putative E-box element in the promoter of HOTAIR, which is recognized by c-Myc. Therefore, c-Myc directly binds to the E-box element and increases the expression levels of HOTAIR. In addition, knockdown of c-Myc transcript reduces both the promoter actrivity and the HOTAIR expression levels, whereas up-regulation of c-Myc gene increases the promoter activity of HOTAIR and its expression levels [46]. Additionally, upon c-Myc activation of HOTAIR expression miRNA-130a levels are down-regulated. A negative association between miRNA-130a and HOTAIR was established in different kind of tumors, such as gallbladder cancerous tissues [47]. Consistently, a recent study showed that in GBM, miR-130a was associated with overall survival of patients treated with TMZ [48]. Collectively, all these data suggests that HOTAIR plays a key role in the initiation and the progression of brain tumors.

3.2. The Functional Role of H19 lncRNA

One of the first lncRNAs that was implicated in tumorigenesis in mammalian cells was H19 as early as 1993 [49, 50]. H19 is widely expressed within endodermal and mesodermal derived tissues during development, while after birth its expression is significantly decreased [51]. As such, H19 has been shown to regulate stemness in hematopoietic/embryonic stem cells [52]. Consistent results have been also observed in the context of GSCs, as H19 is extremely up-regulated in GSCs in comparison to differentiated cells. In addition, in inflammatory and multidrug-resistant tumors, it was demonstrated that up-regulation of H19 occurs during the epithelial-mesenchymal transition (EMT) in both

primary and metastatic tumors [53]. Moreover, Jiang X *et al.* (2016) uncovered the role of H19 in many functions of glioma cells and observed that overexpression of H19 promotes the stemness, invasion, and tumorigenicity of glioblastoma cells [54].

One of the most common properties of solid tumors, including GBM, is hypoxia induction. It has been shown that H19 expression levels are induced in the hypoxic environment of tumors, through direct and indirect actions of HIF1-α (hypoxia-inducible transcription factor 1-α) [55]. Specifically, HIF1-a directly binds to the H19 promoter and up-regulates its expression, leading to increased cell proliferation and tumor growth [55]. Furthermore, HIF1-a is able to induce SP1 (Specific Protein 1), which also targets H19 promoter in order to activate H19 expression under hypoxia [55]. In addition, Matouk and colleagues have reported that p53 induces strong down-regulation of H19 via inhibition of HIF1-a signaling pathway [56]. Moreover, HIF1-a regulates cell proliferation and survival through an autocrine autoregulatory loop with IGF-2 (Insulin Growth Factor 2), a well-studied oncogenic factor in many cancer types. It is established that H19 and IGF-2 expression is constantly coordinated by sharing common regulatory elements within their promoters. In particular, enhancer elements downstream of H19 can coordinately activate the locus of H19-IGF2 genes [57, 58]. All these data suggest that H19 plays a key role in the adaptation of hypoxia stress response in tumor cells.

Finally, H19 is also regulated by c-Myc transcription factor through direct recruitment of c-Myc to H19 gene. Therefore, H19 levels are highly increased in glioma cells, upon direct binding of c-Myc [59]. Conclusively, these data suggest that H19 lncRNA plays a crucial role in GSCs differentiation and cell migration in the brain-derived tumors.

3.3. The Functional Role of CRNDE (Colorectal Neoplasia Differentially Expressed) lncRNA

CRNDE lncRNA was characterized as a novel biomarker for cancer with high expression levels in colorectal tumors. Interestingly, the first association of CRNDE with glioma was in 2012, when Jang *et al.* showed that the most up-regulated lncRNA in glioblastoma is CRNDE (its expression was up-regulated 32 folds in glioblastoma tumors as compared to normal tissue), among differentially expressed lncRNAs. In addition, in the same study the expression levels of CRNDE were closely associated with tumor grade [21]. In another independent study, it was shown that CRDNE has an oncogenic activity, since forced overexpression promotes glioma cell growth and migration, while silencing of CRNDE suppressed the oncogenicity, through the Mammalian Target of Rapamycin (mTOR) signaling pathway. Wang *et al.* observed that upon CRNDE overexpression, the phosphorylation levels of p70S6K -a direct downstream target of mTOR- are dramatically increased leading to induced cell growth and migration both *in vivo* and *in vitro*. On the other hand, knockdown of CRNDE expression caused a decrease in the phosphorylation levels of p70S6K and a concomitant reduction of cell growth and migration, indicating that CRNDE regulates mTOR signaling in glioma [60].

Moreover, a key regulatory role of CRNDE in brain tumors is mediated via epigenetic changes in gene expression with direct binding of chromatin modifying complexes CoREST and PRC2 to CRNDE and histone methylation/demethylation [61]. Additionally, there is evidence that CRNDE is involved in the differentiation of mouse stem cell (moESC). The decreasing levels of CRNDE from birth and onwards suggest a possible connection between CRNDE expression and cell differentiation. Several pluripotency-related transcription factors bind to the CRNDE transcript, suggesting that CRNDE may be involved in the stemness pathway and may affect the GSCs properties [62]. Accordingly, it was observed that EGFR is overexpressed in CRNDE-expressing gliomas, indicating that CRNDE may also regulate the GSCs initiation through the EGFR signaling pathway [63, 64].

3.4. The Functional Role of MALAT1 (Metastasis-associated Lung Adenocarcinoma Transcript 1)

One of the first examples of a lncRNA with involvement in tumor-suppressive functions in the brain is MALAT1 (metastasis-associated lung adenocarcinoma transcript 1). MALAT1 was first reported in glioma in 2016 from Han *et al.* [65]. MALAT1 overexpression in human glioma cell lines and in glioma xenograft models significantly suppresses the cell proliferation and invasion. On the other hand, knock down of MALAT1 enhances these properties of glioma cells both *in vitro* and *in vivo*, suggesting a suppressive function of MALAT1 in glioma progression. In the same study, it was also shown that MALAT1 expression levels were strongly reduced in glioma tumors from human patients in comparison to normal brain tissue. Furthermore, mechanistic studies have shown that overexpression of MALAT1 reduces the levels of ERK1/2 phosphorylation and MMP2/9 expression in U87 and

U251 cells [66]. The kinase/mitogen activated protein kinase (ERK/MAPK) pathway is a crucial signal transduction pathway that regulates cell proliferation and invasion. In addition, inhibition of ERK signaling phenocopies the MALAT1 overexpression-phosphorylation of ERK1/2, suggesting that the tumor-suppressive role of MALAT1 is probably arbitrated via suppression of ERK/MAPK-induced cell growth.

3.5. The Functional Role of MEG3 (Maternally Expressed Gene 3)

MEG3 is associated with prolonged survival in GBM patients [21, 67-68], therefore representing a lncRNA with tumor suppressive functions. Specifically, MEG3 is highly expressed in normal brain, while it is down-regulated in glioma tumors [21, 67]. Wang *et al.* showed that when MEG3 is overexpressed in U87 human cell line, proliferation is inhibited with a significant increase in apoptosis. It was then shown that the anti-proliferative function occurs through the suppression of MDM2 and the subsequent induction of p53 signaling pathway [69]. In addition, by generating a MEG3 knock-out mouse model, Gordon *et al.* showed that MEG3 inhibits vascularization in the brain. Moreover, in these mice, increased levels of VEGF angiogenic pathway associated genes were observed, suggesting the suppressive role of MEG3 in the tumorigenesis through the VEGF signaling cascade [70]. Collectively, these data imply a key role of MEG3 in the GBM initiation and progression as well as its potential usage as therapeutic target in the treatment of glioma.

4. FUNCTIONAL ANALYSIS OF WELL-CHARACTERIZED LNCRNAS IN NEUROBLASTOMA

Recent studies have also proposed regulatory roles for lncRNAs in the initiation and progression of neuroblastoma tumors (Fig. **2**). The most examined paradigms for the role of lncRNAs and their underlying signaling pathways are lncUSMycN and NBAT1.

LncUSMycN is transcribed from the 2p24 chromosomal region, a genomic locus well-correlated with neuroblastoma tumorigenesis [71, 72]. Thus, the amplification of 2q24 has been strongly associated with neuroblastoma and contains numerous coding and non-coding genes with potential contribution to the aggressiveness of this malignancy. The oncogene MYCN is a well characterized gene of the specific locus and it is genetically amplified in 25–30% of neuroblastoma patients. However, one intriguing question that needs to be answered is whether a co-amplification of different lncRNAs and MYCN gene could have additional contribution in neuroblastoma phenotype. In this context, Liu *et al.* have discovered the lncUSMycN as a lncRNA gene 14 kb upstream of the MYCN gene. lncUSMycN is included into the frequently amplified locus on chromosome 2p. Knock-down of lncUSMycN in the neuroblastoma cell line SK-N-BE2 suppresses the expression of MYCN oncogene [73]. Liu and colleagues showed that this regulation of MYCN expression levels was mediated by the RNA binding protein NonO, which interacts with lncUSMycN and regulates MYCN expression at a post-transcriptional level. In addition, xenograft experiments in mice showed that depletion of lncUSMycN caused significant reduction of the tumor volume, while overexpression of the lncRNA in neuroblastoma tumor cell lines had an oncogenic effect, causing increased cell proliferation. In summary, these data suggest that a combination of RNA-protein and protein-protein interactions regulate the expression of MYCN oncogene in a post-transcriptional level and affect the aggressiveness of neuroblastoma cells. Conclusively, it is intriguing to hypothesize that co-amplification of other lncRNAs may also regulate the amplification of MYCN expression.

In addition, SNP rs6939340, a polymorphism linked to high-risk neuroblastoma at the chromosomal locus 6p22, is located in the intronic region of two lncRNAs, NBAT1 and LINC00340 (CASC15). *In vitro* experiments, in a study by Pantley and colleagues, revealed that this risk-related SNP carries an enhancer like properties. Neuroblastoma cells with the risk-related G/G genotype exhibited a lower interaction between the promoter of NBAT1 and the putative enhancer. Moreover, in the same study it was reported that NBAT1 expression is regulated by epigenetic modulators and that in high-risk neuroblastoma patients hypermethylation of NBAT1 promoter was observed [74]. The decreased levels of NBAT1 in high-risk neuroblastoma patients lead to deregulation of NBAT1/EZH2 mediated gene networks. This deregulation enhances proliferation and migration properties of neuroblastoma cells as a result of chromatin structure modifications in the promoters of pro-tumorigenic genes. Taken together, these data suggest that NBAT1 is a key regulator of the progression of neuroblastoma and a promising marker of risk-assessment in neuroblastoma patients.

5. LncRNAs AS POTENTIAL THERAPEUTIC TARGETS FOR NERVOUS SYSTEM-RELATED MALIGNANCIES

HOTAIR is one of the most promising lncRNA for therapeutic targeting in GBM. In particular, by mouse xenografts studies, it was shown that reduction of HOTAIR expression levels led to significant repression of human tumor growth [26, 45], proposing that targeting this lncRNA could be a potential therapeutic strategy. Thus, HOTAIR is one of the first lncRNA that has been targeted by a specific molecule. Specifically, treatment with I-BET151, a BET bromodomain inhibitor, reduces the expression of HOTAIR, which is consistent with the previous findings that BRD4, an I-BET151 target, directly enhances HOTAIR gene expression (Fig. **1B**) [26]. Moreover, treatment with I-BET151 impairs cell proliferation *in vitro* and *in vivo* in GBM cell lines and patient-derived cells [26]. BET proteins are modulators of epigenetic pathways, up-regulated in GBM and promising therapeutic targets in various cancers [75, 76]. Most importantly, overexpression of HOTAIR nullifies the effects of I-BET151 treatment in GBM cells, suggesting not only that HOTAIR expression is controlled by BET proteins, but also that the inhibition of GBM growth by I-BET151 may be mediated by HOTAIR. These observations validate the use of HOTAIR as a promising target for GBM tumors.

In addition, MALAT1 lncRNA has a specific implication in the chemoresistance of glioma cells to TMZ. MALAT1 is dramatically suppressed in patients responding to TMZ chemotherapy as compared to non-responding patients. Moreover, MALAT1 is overexpressed in TMZ-resistant glioma cell lines as compared to the parental cells, while upon silencing it partially reverses this chemoresistance effect, probably through miR-203 regulation. Indeed, miR-203 enhances TMZ cytotoxicity and re-sensitizes resistant glioma cells through direct targeting of thymidylate synthase (TS), a crucial regulator of cell proliferation, cell cycle progression and target of various chemotherapeutics. Overall, MALAT1 not only induces TMZ chemoresistance, but also its inhibition reverses this effect, mediated by up-regulated miR-203 and suppressed TS expression [77]. Other studies reveal that siRNA against MALAT1 sensitizes the chemoresistant glioma cell lines by down-regulating ZEB1, an EMT related protein [78]. These data about the role of MALAT1 in tumor cell infiltration and TMZ chemoresistance led to the development of a nanocomplex against MALAT1, composed by DOTAP/DOPE liposomes, single-chain antibodies against the transferrin receptor (TfRscFv) and siRNAs against MALAT1. This nanocomplex permeates the blood-brain barrier and shows impressive results in suppressing proliferation, motility and stem properties of GBM cells in animal models of highly TMZ- resistant GBM [79].

Another example for potential therapeutic targeting in nervous system malignancies is XIST, one of the most extensively studied lncRNAs. XIST is necessary for the viability of many GBM cell lines and it is linked to DNA synthesis capability, a very important factor for the resistance to TMZ. Accordingly, XIST may enhance the chemoresistance of glioma cells to TMZ since XIST knock-down sensitizes glioma cells to TMZ [80] and significantly reduces its IC50 values [81]. XIST promotes the chemoresistance of glioma cells to TMZ, by direct binding and inhibition of miR-29c [81], a well-characterized tumor suppressor in lung cancer [82, 83]. The importance of miR-29c on the XIST-mediated chemoresistance of glioma cells is further supported by the increase of IC50 for TMZ after miR-29c inhibition.

Another therapeutic opportunity is provided by the TALNEC2 lncRNA. TALNEC2 is highly expressed in GBM tumors and its expression levels are correlated with the aggressiveness of GBM cells [84]. In agreement, TALNEC2 is also highly expressed in GSCs and regulates their stemness (Nanog, SOX2, Oct4), mesenchymal transformation (CTGF, fibronectin, YKL40) and migration [85]. Mesenchymal transformation is associated with cell infiltration and resistance to radiotherapy [86, 87]. Consistently, TALNEC2-silenced GSCs show decreased proliferation and much higher sensitivity to γ-radiation. Moreover, silencing of TALNEC2 prolongs the survival of mice bearing intracranial xenografts of GSCs [85].

Furthermore, extra therapeutic opportunities for central nervous system tumors and especially GBM are provided by the connection and cross-regulation of lncRNAs with miRNAs. For example, linc00152 is expressed in many cancers and is associated with poor survival of patients [88]. It affects the expression of miR-103a-3p as a competing endogenous RNA [89]. miR-193a-3p has been also identified as prognostic biomarker in various cancers [90, 91]. Specifically in glioma tumors and GSCs, linc00152 is up-regulated and miR-103a-3p is down-regulated. *In vitro* studies showed that linc00152 functions as an oncogene in GSCs, being important for the proliferation, migration and invasion of the GSCs. In contrast, miR-103a-3p functions as a tumor-suppressor in GSCs, delaying

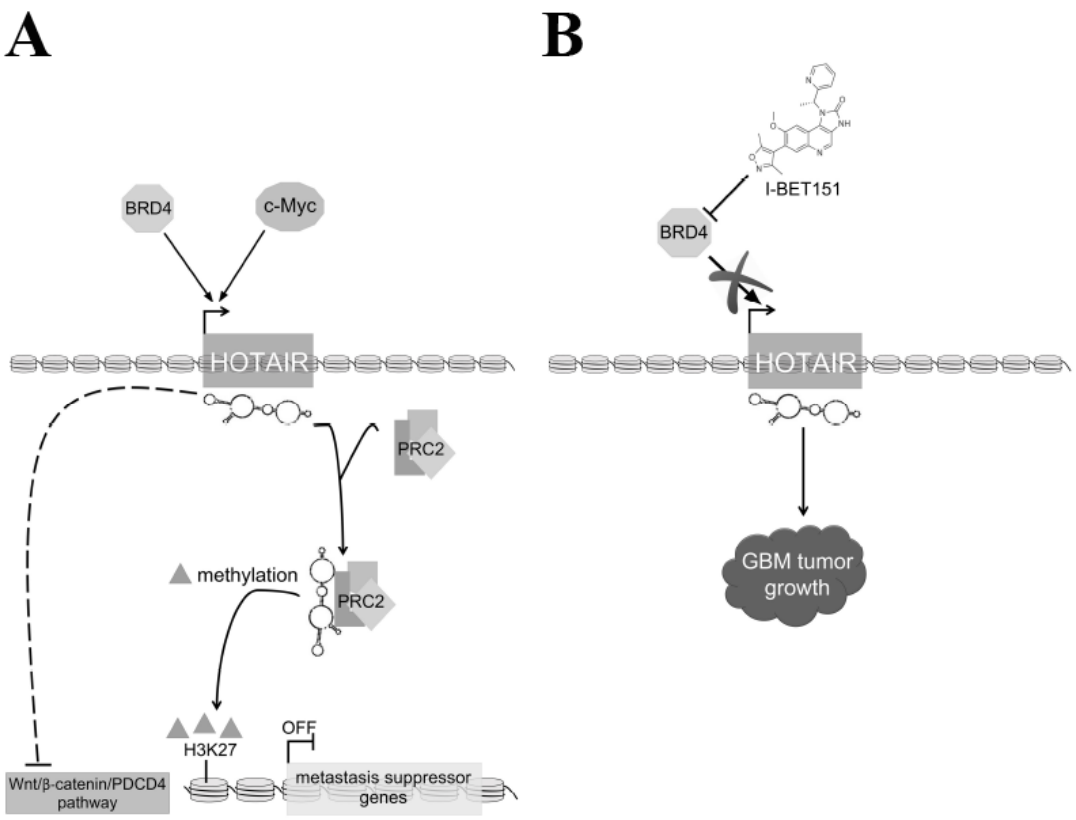

Fig. (1). Schematic depiction of the functional role and targeting of lncRNA HOTAIR in GBM. (A) HOTAIR acts as an oncogene in GBM. It is directly regulated by BRD4, a BET domain epigenetic modulator and can also be activated by c-Myc. HOTAIR binds directly to PRC2, a chromatin modifying complex, promoting the histone H3K27 trimethylation and leading to epigenetic silencing of metastasis suppressor genes. Additionally, it is able to inhibit the Wnt/β-catenin/PDCD4 pathways, leading to induction of cell cycle arrest and suppression of tumor metastasis. (B) I-BET151 is a BET protein inhibitor. It targets BRD4 so that it cannot enhance the HOTAIR gene expression, leading to inhibition of GBM tumor growth.

their sphere formation ability and promoting apoptosis. Interestingly, bioinformatic analysis predicted that linc00152 is a direct target of miR-103a-3p. In agreement, the effects of linc00152 knockdown on GSCs are arbitrated by miR-103a-3p resulting in tumor suppression. Tumor xenografts study revealed that linc00152 knockdown together with miR-103a-3p overexpression caused a significant decrease in the tumor growth and exhibited high survival in nude mice [92].

Collectively, these observations illustrate the potential use of lncRNAs in anti-cancer therapies (Fig. **2**). However, these studies constitute the first steps towards this direction and much more research efforts are needed to accomplish this ambitious goal.

6. LncRNAs AS NOVEL BIOMARKERS OF NERVOUS SYSTEM CANCERS

Multiple studies indicate that numerous lncRNAs alter significantly their expression patterns in body fluids and cancer tissues (Fig. **2**) [93]. These alterations provide new insights for the utilization of lncRNAs as novel biomarkers for cancer diagnosis and prognosis. Therefore, lncRNAs could be used as specific molecular indicators of the disease status in nervous system malignancies, detectable in body fluids, such as serum or urine (circulating lncRNAs), or detectable in cancerous nervous tissue (non circulating lncRNAs), and can be important for the detection and management of these diseases (Table **1**).

6.1. Circulating lncRNAs as Biomarkers

Circulating nucleic acids (CNAs), including lncRNAs, are correlated to tumor burden and malignant progression, thus indicating their potential use as tumor biomarkers for diagnosis and tumor prognosis, easily detected by PCR assays [94]. Despite the fact that the functional role of lncRNAs has been clearly established, their usage as biomarkers in nervous system cancers is not well characterized [95]. Only recently, it was reported that serum analysis from glioblastoma (GBM) patients indicates that HOTAIR and GAS5 lev-

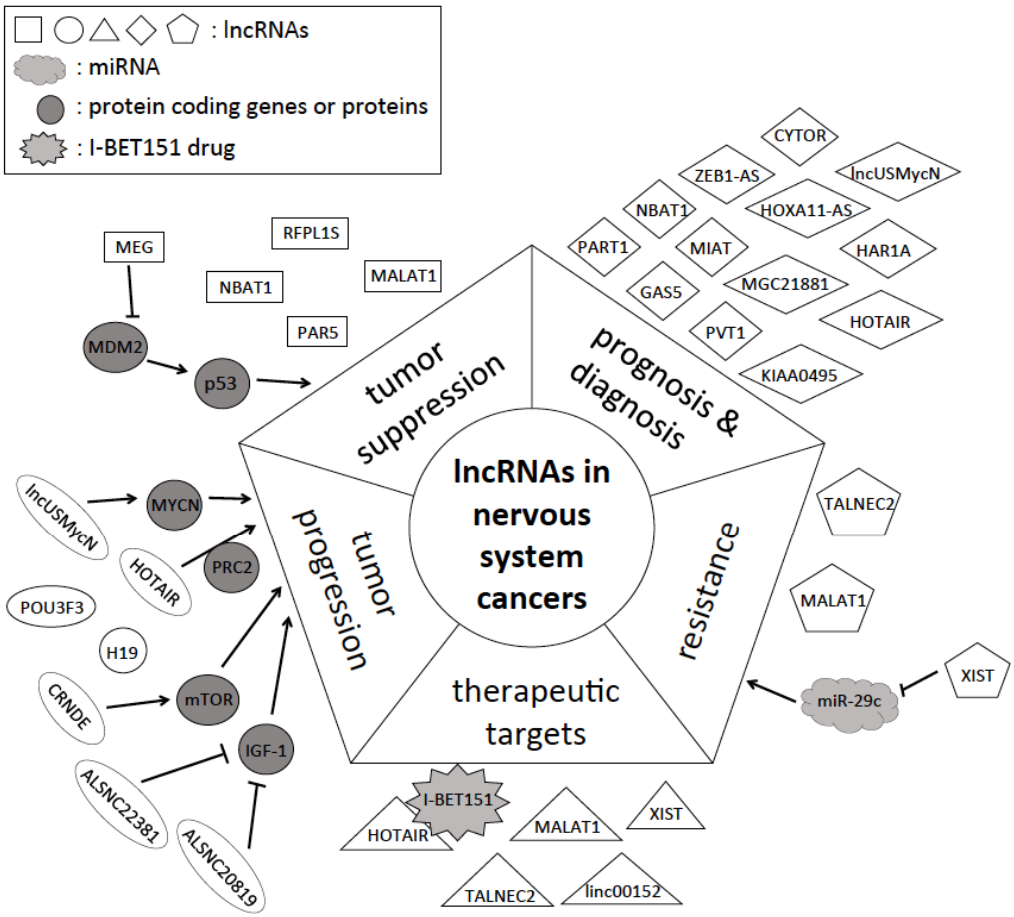

Fig. (2). Graphical abstract of lncRNAs' roles in nervous system malignancies. LncRNAs are represented as white shapes, miRNA as grey cloud and protein coding genes or protein molecules as dark grey circular shapes. Inductive actions are indicated with arrows and suppressive actions with repression symbols.

Table 1. LncRNAs as biomarkers in nervous system malignancies.

LncRNAs	Type of nervous system malignancy	Circulating/non-circulating	Changes	Refs.
HOTAIR	*GBM*	*Serum circulating*	*Up*	[95]
GAS5	*GBM*	*Serum circulating*	*Down*	[95]
HOXA11-AS	*GBM*	*Tumor*	*Up*	[98]
ZEB1-AS1	*GBM*	*Tumor*	*Up*	[99]
PVT1	*GBM*	*Tumor*	*Up*	[95]
CYTOR	*GBM*	*Tumor*	*Up*	[95]
HAR1A	*GBM*	*Tumor*	*Down*	[95]
MIAT	*GBM*	*Tumor*	*Down*	[95]
MALAT-1	*GBM*	*Tumor*	*Up*	[32]
NEAT	*GBM*	*Tumor*	*Up*	[103]
CACS2	*GBM*	*Tumor*	*Down*	[105]
NBAT1	*NB*	*Tumor*	*Down*	[37, 74]
LncUSMycN	*NB*	*Tumor*	*Up*	[73]

GBM: Glioblastoma, NB : Neuroblastoma

els are associated with survival rate [95]. In particular, elevated expression levels of HOTAIR are associated with tumor progression and lethality, whereas high levels of GAS5 indicate a different pattern being linked to reduced rates of tumor growth and lethality [95]. Moreover, the same study showed a worse overall survival outcome for patients with a combination of high HOTAIR and low GAS5 levels, as compared to patients with low HOTAIR and high GAS5 levels. These data establish both HOTAIR and GAS5 lncRNAs as prognostic biomarkers of survival rates and cancer progression in patients with glioblastoma. This study provides a nice paradigm of utilization of lncRNAs as circulation biomarkers in glioblastoma, underscoring the need for additional research efforts in this context (Table **1**).

6.2. Non Circulating lncRNAs as Biomarkers

LncRNAs are involved in several oncogenic and tumor suppressive pathways and, their expression levels in cancerous tissue are correlated with favorable or poor prognosis, making them promising prognostic biomarkers. One of the well-characterized lncRNAs that act as a tumor prognostic factor is HOTAIR (Table **1**). Accordingly, HOTAIR expression is tightly linked with metastasis and poor survival prognosis [40, 96]. Recent studies have also shown that HOTAIR expression is significantly up-regulated in high grade gliomas in comparison with low grade or healthy brain tissues. Moreover, a Kaplan-Meier survival analysis indicated a negative correlation between HOTAIR expression levels and overall survival rates in glioma patients [97].

In addition, another example of an extensively studied lncRNA in cancer biology is HOXA11-AS (Table **1**). It has been recently reported that high expression levels of HOXA11-AS are observed in glioma tumors, compared to normal tissue. In addition, HOXA11-AS may also be implicated in the progression of this tumor, as it is mainly found in high grade glioma samples [98]. Using the TCGA (The Cancer Genome Atlas) database for Kaplan-Meier analysis, this study showed that high expression of HOXA11-AS is correlated to poor survival outcome, demonstrating the prognostic value and future therapeutic potential of this lncRNA [98].

Another paradigm is ZEB1-AS1 (Table **1**), which plays an important role as a novel prognostic biomarker in GBM. It has been recently shown by qRT-PCR that the expression levels of ZEB-AS1 are significantly up-regulated in gliomas [99]. Furthermore, analysis of 82 glioma patients indicated that ZEB1-AS1 is highly expressed in high grade tumors as compared to low grade ones, providing evidence that ZEB1-AS1 is a key regulator in GMB progression. Finally, Kaplan-Meier analysis in these 82 patients suggests a poor overall survival rate in patients with high levels of ZEB1-AS1 [99].

It was also shown by two independent recent studies that the lncRNAs PVT1 (Pvt1 oncogene) and CYTOR (cytoskeleton regulator RNA) are up-regulated in high grade GBM tumors when compared to lower grade GBMs (LGGs) [95]. In the same study, an opposite expression pattern was observed for HAR1A (highly accelerated region 1A) and MIAT (myocardial infarction associated transcript), showing significantly lower levels in GBM samples than LGGs [95]. In independent long-rank analyses of TCGA and GEO data, high PVT1 expression and low HAR1A expression were correlated with poor survival outcome in glioma patients [95]. Besides that, both PVT1 and HAR1A can play a key role as markers of anti-cancer therapy response. Two different groups of GBM patients were studied for their response in chemotherapy and radiotherapy. The group of patients presenting low PVT1 and high HAR1A expression was associated with significantly better outcome as compared to the group with high PVT1 and low HAR1A expression levels [95]. Moreover, PVT1 and HAR1A play a significant role in the progression of GBM tumorigenesis and are not only suitable as prognostic biomarkers, but also as indicators for the patients' response to the cancer therapy.

Moreover, a number of other studies connect lncRNAs with GBM by uncovering many examples of differentially expressed lncRNAs that are associated with the survival rates and therefore, could serve as biomarkers for GBM diagnosis and prognosis [100]. There are specific lncRNAs such as ZEB1-AS11 [13], linc-OIP5 [101], AB073614 [102], CRNDE [32, 100], XIST [103], NEAT [103] and MALAT1 [32] that are highly expressed in glioma tumors and Cox regression analysis has indicated correlation between high levels and poor survival progression. On the other hand, lncRNAs such as MEG3 [100], MIAT [104] and CACS2 [105] display low expression pattern in GBM malignancy and indicate better survival outcome.

Furthermore, lncRNAs expression levels have been tightly connected to neuroblastoma diagnosis and prognosis. For example, NBAT1 and lncUSMycN have been studied for their clinical importance and may be used as predictors of risk assessment in neuroblastoma patients (Table **1**) [8, 71, 73-74]. Importantly, NBAT1

acts as an independent predictor of survival in high-risk patients and it shows lower expression in all high-risk neuroblastoma subtypes. Conclusively, further investigation in the expression patterns of lncRNAs in body fluids and tumors would increase their diagnostic value, since lncRNAs are relatively stable molecules and very easily detected with high sensitivity.

CONCLUDING REMARKS

LncRNAs represent a new exciting frontier in molecular oncology with emerging roles in the initiation, progression and outcome of nervous system-related malignancies (Fig. **2**). Recent functional genomics studies indicate that lncRNAs demonstrate differential expression patterns in nervous system tumors and are remarkably connected to better overall survival outcome. Numerous differentially expressed lncRNAs are associated with major oncogenic or suppressive signaling pathways in these malignancies, affecting multiple functions of tumor cells.

Generally, lncRNAs act either through modification of chromatin organization or post-transcriptional regulation. In nervous system cancer biology, interaction of lncRNAs with epigenetic modulators is associated with oncogenic activity through alterations of the chromatin organization and subsequent silencing of tumor suppressor genes. On the other hand, the post-transcriptional regulation mediated by lncRNAs affects major signaling pathways or crucial miRNAs for the tumor initiation, progression and metastasis, resulting in oncogenic or tumor suppressive events, depending on each specific lncRNA.

However, this field is in its infancy as it is still not clear enough whether the differential expression of lncRNAs in nervous system malignancies is sufficient for tumorigenesis or it is just merely a consequence of the tumors themselves. In this context, overexpression or knock-down approaches by direct targeting of specific lncRNAs results in significant modulation of tumorigenic properties of glioma or neuroblastoma cells, including cell growth, migration and invasion. These observations suggest a causative role of specific lncRNAs in nervous system-related cancers. However, the functional role for the majority of the affected lncRNAs in gliomas and neuroblastomas is yet not fully understood. Therefore, it is of paramount importance to further investigate the functions and clinical applications of these molecules in nervous system malignancies.

Few pioneering studies show the clinical significance of lncRNAs in GBM. There are already two well-studied lncRNAs for their potential therapeutic role. In combination with the established therapeutic scheme based on TMZ treatment, these targets (MALAT1 and HOTAIR) could be utilized for better clinical outcome in GBM therapy. In addition, many lncRNAs may serve as reliable biomarkers for disease detection, progression and management. LncRNAs such as HOTAIR and GAS5 are already used as biomarkers in GBM patients, as they are stable and easily detectable in serum, allowing a noninvasive diagnosis. As far as non-circulating biomarkers are concerned, the expression levels of a number of lncRNAs, such as PVT1 and HAR1A, in GBM samples can provide reliable markers for glioma patients' prognosis. Finally, the role of lncRNAs in other types of brain malignancies, such as meningiomas, medulloblastomas and gangliogliomas, needs further investigation, since their function and clinical applications in these gliomas are apparently underscored. Understanding how these non-coding transcripts regulate tumorigenesis in the central and peripheral nervous system could provide new promising therapeutic opportunities.

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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ORIGINAL ARTICLE

Prox1 inhibits neurite outgrowth during central nervous system development

Valeria Kaltezioti[1] · Iosifina P. Foskolou[1] · Matthieu D. Lavigne[2] · Elpinickie Ninou[1] · Matina Tsampoula[1] · Maria Fousteri[2] · Marigoula Margarity[3] · Panagiotis K. Politis[1]

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Abstract

During central nervous system (CNS) development, proper and timely induction of neurite elongation is critical for generating functional, mature neurons, and neuronal networks. Despite the wealth of information on the action of extracellular cues, little is known about the intrinsic gene regulatory factors that control this developmental decision. Here, we report the identification of Prox1, a homeobox transcription factor, as a key player in inhibiting neurite elongation. Although Prox1 promotes acquisition of early neuronal identity and is expressed in nascent post-mitotic neurons, it is heavily down-regulated in the majority of terminally differentiated neurons, indicating a regulatory role in delaying neurite outgrowth in newly formed neurons. Consistently, we show that Prox1 is sufficient to inhibit neurite extension in mouse and human neuroblastoma cell lines. More importantly, Prox1 overexpression suppresses neurite elongation in primary neuronal cultures as well as in the developing mouse brain, while Prox1 knock-down promotes neurite outgrowth. Mechanistically, RNA-Seq analysis reveals that Prox1 affects critical pathways for neuronal maturation and neurite extension. Interestingly, Prox1 strongly inhibits many components of Ca^{2+} signaling pathway, an important mediator of neurite extension and neuronal maturation. In accordance, Prox1 represses Ca^{2+} entry upon KCl-mediated depolarization and reduces CREB phosphorylation. These observations suggest that Prox1 acts as a potent suppressor of neurite outgrowth by inhibiting Ca^{2+} signaling pathway. This action may provide the appropriate time window for nascent neurons to find the correct position in the CNS prior to initiation of neurites and axon elongation.

Keywords Axon extension · Calcium signaling · CamkII · CREB phosphorylation · Neuronal maturation

Introduction

One of the most challenging endeavors of biomedical research is to unravel the molecular mechanisms that regulate diversity, specification, and maturation of neural cells during embryonic development. Failure of neuronal progenitors or early born neurons to make the correct differentiation and maturation decisions can lead to developmental impairments, malformations, and neurological diseases or tumors. Neurite and axon outgrowth is a critical step for neuronal maturation. Despite the wealth of information on the role of extracellular factors and signaling pathways in neurite outgrowth [1, 2], little is known about the intrinsic gene regulatory factors that control this process during development.

In this regard, here, we report the identification of Prox1, a homeobox transcription factor, as a key player in the gene regulatory networks that negatively control neurite elongation. Prox1 is a crucial regulator for embryonic development

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✉ Panagiotis K. Politis
ppolitis@bioacademy.gr

[1] Center for Basic Research, Biomedical Research Foundation of the Academy of Athens, 4 Soranou Efesiou Street, 115 27 Athens, Greece

[2] Institute for Fundamental Biomedical Research, BSRC 'Alexander Fleming', 34 Fleming Street, Vari, 16672 Athens, Greece

[3] Laboratory of Human and Animal Physiology, Department of Biology, School of Natural Sciences, University of Patras, 26500 Rio Achaias, Greece

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and morphogenesis of various organs such as liver, pancreas, heart, intestinal, lymphatic and hematopoietic systems, lens, retina, brain, and spinal cord [3–14]. Therefore, mouse embryos lacking *Prox1* die during development at embryonic day 14.5 (E14.5), exhibiting many developmental defects [10, 14, 15]. Interestingly, Prox1 has been previously reported to affect gene expression during organ development and homeostasis through its ability to interact with many other transcription regulators and to function as a transcriptional co-repressor [6, 16–26]. Moreover, Prox1 acts both as tumor suppressor and oncogene during cancer development and progression, depending on the cell type or tissue of tumor origin [27–34].

Prox1 has important and diverse roles in central nervous system (CNS) development. In particular, Prox1 promotes neurogenesis and inhibits astrogliogenesis and self-renewal of neural stem cells [6]. Additionally, Prox1 exerts a negative action in motor neuron generation in the ventral spinal cord by suppression of Olig2, the key regulator of motor neuron progenitor domain [7]. Furthermore, Prox1 promotes the migration and differentiation of caudal ganglionic eminence (CGE)-derived interneurons during cortical development [35, 36]. Moreover, Prox1 inhibits cell cycle progression and proliferation of neuroblastoma cancer cells via a direct action in basic components of the cell cycle machinery [30]. Finally, Prox1 acts as a major regulator of cell fate identity and adult neurogenesis in the hippocampus [37, 38].

Although Prox1 expression is maintained in specific neuronal sub-types in postnatal or adult CNS, in most neuronal sub-types, Prox1 is only transiently expressed during development in the stage of neuronal progenitor or early born neuron. Then, Prox1 expression is normally down-regulated in mature, terminally differentiated neurons, prior to neurite and axon extension [4–7, 35, 36, 39, 40]. Together, these observations led to the hypothesis that Prox1 may exert a negative effect on neurite extension and axon outgrowth, in general, with important consequences in nervous system development. In agreement with such a role, human *PROX1* gene locus has been associated via genome-wide association studies (GWAS) with neurological and neurodevelopmental diseases, including Schizophrenia [41, 42] and Alzheimer's disease [43].

Here, we provide functional evidence that Prox1 is indeed a critical regulator of neurite elongation and neuronal maturation. In particular, we show that Prox1 expression prevents neurite extension in neuroblastoma cell lines and primary neurons and suppresses many genes involved in neurite and axon outgrowth pathways as well as terminal neuronal identity. Interestingly, Prox1 represses many components of Ca^{2+} signaling pathway, an important mediator of neuronal maturation processes [44–46], and as a result, it negatively affects Ca^{2+} influx in mouse embryonic neurons. Collectively, our data give an insight on how Prox1 regulates neurite outgrowth, to allow timely controlled activation of the molecular changes needed for neuronal maturation.

Materials and methods

Cell culture of cell lines and primary cells

Neuroblastoma cell lines were cultured as previously described [6, 30, 47]. In brief, the mouse neuroblastoma Neuro2A cells were cultured in DMEM with 1gr/L glycose supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin. For maintenance of stable cell line selection, G418 (300 μg/mL) and hygromycine B (200 μg/ml) were used and purchased from Calbiochem and AppliChem, respectively. The human neuroblastoma cell line SH-SY5Y was cultured in DMEM/F-12 (1:1) with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin. Retinoic acid (RA)-induced neurite extension was performed by adding RA (20 μM) in culture media and incubating the cells for 4 days.

Forebrain and spinal cord neurons were isolated from mice embryos at E15.5 and E14.5, respectively, dissociated by trypsinization (0.25% Trypsin/EDTA, Sigma) for 10 min at 37 °C, and triturated into single-cell suspension. Cells were cultured in neurobasal medium (Gibco, Invitrogen) supplemented with 2% B27 (Invitrogen) and 0.2% insulin (Gibco), 2 mM L-glutamine, and 1% penicillin/streptomycin.

Overexpression studies in Neuro2A and SH-SY5Y cell lines

Overexpression studies in Neuro2A and SH-SY5Y cells were performed with transient transfections using Lipofectamine 2000 (Invitrogen) or $CaCl_2$ method with HEPES (Fluka), as previously described [6, 30, 47, 48]. Plasmids used for transfections were previously described [7, 30]. Construction of the inducible Prox1-Tet-On and GFP-Tet-On Neuro2A stable cell lines were previously described [30] and transgene expression was turned on with Dox (1 μg/mL; AppliChem). Prox1 induction after adding Dox (added for 2 days) was confirmed by immunofluorescence. Prox1 overexpression and GFP expression in Tet-On inducible Neuro2A were further verified by western blot using rabbit anti-Prox1 (102-PA32, ReliaTech, 1:1000 dilution), rabbit anti-GFP (Invitrogen, 1:1000 dilution), and, as an internal control, mouse anti-β-actin (A1978, Sigma, 1:15.000 dilution).

Overexpression and knock-down studies in primary neuronal cells

Overexpression studies in E15.5 embryonic forebrain primary neurons and E14.5 embryonic spinal cord neurons

were performed using *pCAGGS-mProx1-IRES-GFP* or *pCAGGS-IRES-GFP*, *as a control* vector. The *pCAGGS-mProx1-IRES-GFP* vector was constructed by transferring the open-reading frame of Prox1 from *pcDNA3.1-mProx1* described in [6, 7] to *pCAGGS-IRES-GFP* (a generous gift from Stavros Taraviras, Medical School, University of Patras, [49]. The forced overexpression of Prox1 or GFP was performed using Amaxa electroporator (Lonza). For each electroporation 6 µg of plasmid DNA was used, as previously described [6, 7, 50]. For rescue experiments described in Supplementary Figure S6, VP16-CREB construct, a constitutively active form of CREB, was used. This plasmid was a generous gift from Dr. Beatriz del Blanco and Prof. Angel Barco (Instituto de Neurociencias de Alicante, Spain) and described in [51].

Knock-down studies in E14.5 embryonic ventral forebrain primary neurons were performed using shRNA plasmid constructs expressing GFP together with *shProx1* (shProx1-GFP) or *shSCR* (shSCR-GFP), as previously described [7]. The expression of shRNA constructs in primary neuronal cultures was achieved by Amaxa electroporator (Lonza). For each electroporation, 6 µg of plasmid DNA was used [6, 7, 50].

Immunofluorescence analysis

On tissue sections

Mouse embryos were fixed with 4% paraformaldehyde (PFA) solution for 5 h (E10.5) or 18 h (E14.5), and cryoprotected in 30% sucrose, frozen, and cut in 14-µm-thick sections in cryostat. Sections were blocked with 0.1% Triton X-100 and 5% fetal bovine serum (FBS) in PBS for 30 min and were subsequently incubated with primary antibodies at 4 °C overnight. Sections were washed and incubated at room temperature (RT) for 1 h with the appropriate secondary antibodies. Cell nuclei were labeled with DAPI (1:2000, Molecular Probes). Specimens were viewed and analyzed with confocal microscopy (Leica TCS SP5 on a DMI6000 inverted microscope) by keeping the same settings between experiments. For BrdU experiments, we injected E9 pregnant mice (2 injections with 2-h interval) with BrdU 50 mg/kg (body weight, per single injection). To detect BrdU, cryosections were first treated with 2 M HCl, 0.1% Triton-100, for 30 min, at 37 °C and then with 0.1 M sodium borate, pH = 8.5, for 20 min, at RT, as previously described [6, 48]. The number of triple-positive (Prox1 + /BrdU + /bIII-Tub +) cells versus double-positive (BrdU + /bIII-Tub +) cells was estimated using Adobe Photoshop software. In particular, using the Photoshop count tool, we first marked and measured the double-positive cells. Then, we manually identified which of these cells are positive for Prox1 (triple-positive) by adding the green layer (Prox1 signal) on the top of red (BrdU signal) and gray (bIII-Tub signal) layers. In total, we measured all cells from 5 independent sections, obtained from three different mouse embryos (5 sections per embryo).

On cultured cells

Cells were placed on poly-L-lysine-coated cover slides (13 mm), fixed in 4% PFA, and then, we followed the same procedure as tissue sections. The detection of CREB phosphorylation was performed by immunostaining of E15.5 forebrain neurons, which were first AMAXA electroporated and then cultured for 7 days. To induce CREB phosphorylation, these cells were pre-cultured for 1 h in a low K^+/Ca^{2+} buffer (129 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM glucose, 0.1% BSA, and 25 mM HEPES [pH 7.4]) and then stimulated by the addition of 50 mM KCl solution prior to fixation and immunostaining. KCl solution was used to depolarize the neuronal cells, induce calcium influx, and finally promote CREB phosphorylation [52–54]. The % of pCREB cells was estimated by measuring the number of double-positive (pCREB + + /GFP +) cells versus the number of GFP + only cells, by using Adobe Photoshop software. In particular, using the Photoshop count tool, we first marked and measured the GFP + cells. Then, we manually identified which of these cells are positive for pCREB (double-positive) by adding the red layer (pCREB signal) on the top of green (GFP signal) layer. In total, we measured cells from 4 independent cultures.

Prox1 was detected using a rabbit polyclonal anti-Prox1 antibody from ReliaTech (102-PA32, 1:100 dilution), and GFP was detected using a rabbit polyclonal anti-GFP antibody (A-6455, Invitrogen, 1:500 dilution) or chicken anti-GFP (Ab13970, Abcam, 1: 4000). Mouse monoclonal anti-bIII-tubulin was from Covance (USA) (MMS-435P-250, 1:1500 dilution) and rabbit polyclonal TAU was from Cell Signaling (46687S, 1:100 dilution). Rat anti-BrdU monoclonal antibody was purchased from Abcam (Ab6326, 1:400 dilution). Rabbit anti-pCREB was provided from Cell signaling (9198, 1:300 dilution), and for rescue experiments with VP16-CREB vector, the rabbit anti-VP16 tag was used from Abcam (Ab 4808, 1:500 dilution). Secondary antibodies conjugated with AlexaFluor 488 (green), 568 or 594 (red), and 647 (far red) were from Molecular Probes (1:500 dilutions).

Neurite length measurement

To measure the neurite length of neuroblastoma cells and primary neurons, cells were seeded on coverslips coated with poly-L-lysine (Sigma) and cultured for 4 days with RA in the case of neuroblastoma cells, or 4 days with neurobasal medium in the case of primary neurons. Then, cells were fixed in 4% PFA, immunostained with mouse

anti- bIII-tubulin in Neuro2A whereas in primary cells with mouse anti-bIII-tubulin and rabbit anti-Tau, an axonal marker. Then, cells were analyzed with confocal microscopy. The length of the longest neurite of cells expressing the transgene was measured using ImageJ software. Specifically, neurite outgrowth was estimated by manually tracing the length of the longest neurite. We measured all cells in a field that had an identifiable neurite, respectively, and for which the entire neurite could be visualized. The length of the neurite was determined from the soma to the end of the projection. The length of neurites was determined in micrometers (μm).

In utero electroporation (IUE)

IUE was performed in mouse embryos E14.5 as previously described [49, 55]. Pregnant mice were anesthetized using isoflurane. Then, 1–1.5 μg of plasmids (1 μg/μl) were mixed with 0.01% Fast Green (Sigma) and microinjected into the lateral ventricle of the forebrain of E14.5 mouse embryos. For Prox1 overexpression, *pCAGGS-mProx1* was mixed with *pCAGGS-GFP* vector in a ratio 3:1. For control electroporations, *pCAGGs empty* vector and *pCAGGS-GFP* vector were mixed in the same ratio. Embryonic brains were electroporated using an electroporator (BTX ECM830) with five 40-V pulses (50 ms duration; 950 ms interval). Three days after electroporation, the brains from the embryos were obtained (E17.5), fixed for 16 h in 4%PFA at 4° C. After washing in PBS, they were cut in 100-μm-thick sections in vibrotome (Leica VT1000 S) and were used for immunostaining experiments. Then, the immunostained sections were analyzed with confocal microscopy and the length of neurites was measured using IMARIS Filament tracer module, version 9.1.2 (Bitlane an Oxford Instruments company Inc), following the double-positive GFP + /Tau + neurons.

This study protocol was approved by the local ethics committee (Athens Prefecture Veterinarian Service; 237,208/31-03-2020) and took place in the animal facilities of the Center for Experimental Surgery of the Biomedical Research Foundation of the Academy of Athens. All animals were handled in strict accordance with good animal practice as defined by the relevant U.S. (Office of Laboratory Animal Welfare-NIH) and Greek animal welfare bodies.

RNA extraction and real-time RT-PCR analysis

Total RNA was isolated using the TRI reagent solution (Sigma) followed by treatment with RQ1 DNase (Promega). RNA concentration was measured by Nanodrop 2000c (ThermoFisher Scientific) and 1 μg of RNA was used for cDNA synthesis using the M-MLV Reverse Transcriptase (ThermoFisher Scientific) together with random hexamer primers. Quantitative real-time RT-PCR analysis was performed in a LightCycler® 96 Instrument (Roche), as previously described [6, 7]. Measured values were normalized using geometric mean of m*β-actin* and *mRpl13a* levels.

Primer sets used in RT-PCR assays:
mCamk2a-For: AATGGCAGATCGTCCACTTC
mCamk2a-Rev: GGGTGATCTGACAGGGAGAA
mCamk2b-For: GTACCAGCCAGTCCGAAGAG
mCamk2b-Rev: GGCTCCAAACACCAACTCTG
mCamkk2-For: TCATGTGTCTCTAGCCAGCC
mCamkk2-Rev: TGACCACGATGAAGGATTCCAT
mRara-For: CTCATCTGTGGAGACCGACA
mRara-Rev: ATGCTCCGAAGGTCTGTGAT
mCdk5-For: ACCTGGACCCTGAGATTGTG
mCdk5-Rev: CCCCATTCCTGTTTATGAGC
mCamk4-For: ACCCAGAAGCCCTATGCTCT
mCamk4-Rev: GTGTGAGAGACGCAGGAGAA
mCacnb3-For: CAGGGCTCTGGAGTCAACTT
mCacnb3-Rev: GTCTCCGCCCTCTTTCACTA
mCacnb1-For: GGTCAAACTGGACAGCCTTC
mCacnb1-Rev: CTCCCAGACTGGAACTGGAG
mNos1-For: CCTCGCTTCCTTAAGGTCAA
mNos1-Rev: GCGGACATCTTCTGACTTCC
m Arc-For: GAGCCTACAGAGCCAGGAGA
m Arc-Rev: ACCCATGTAGGCAGCTTCAG
mScn1b-For: TGGCAGAGATGGTGTACTGC
mScn1b-Rev: CCTGGACGCCTGTACAGTTT
mScn3a-For: ATGGAGCACTACCCGATGAC
mScn3a-Rev: ATTCCAGCCCTCTTGGAAAT
mScn3b-For: AGAAAAGATGCCTGCCTTCA
mScn3b-Rev: GATGCATCTCAGCTTCATGG
mScn9a-For: TGCTGTCTCCCTTCAGTCCT
mScn9a-Rev: CAATCTGGAGGGTTGCTCAT
mβ-actin-For: CCCAGGCATTGCTGACAGG
mβ-actin-Rev: TGGAAGGTGGACAGTGAGGC
mRpl13a-For: TTCGGCTGAAGCCTACCAGAAAGT
mRpl13a-Rev: TCTTCCGATAGTGCATCTTGGCCT

RNA-sequencing

Total RNA was isolated from mouse Neuro2A Prox1 and GFP overexpression cells (GFP-Tet-On and Prox1-Tet-On Neuro2A cells) using Trizol (ThermoFisher Scientific). The quantity and quality of RNA samples were analyzed by the BSRC 'Alexander Fleming' Genomics Facility using Agilent RNA 6000 Nano kit with the bioanalyzer from Agilent. RNA samples with RNA Integrity Number (RIN) > 7 were used for library construction using the 3′ mRNA-Seq Library Prep Kit Protocol for Ion Torrent (QuantSeq-LEXOGEN™) according to manufacturer's instructions. DNA High Sensitivity Kit was used with the bioanalyzer to assess the quantity and quality of libraries. Libraries were

then pooled and templated using the Ion PI™ HiQ OT2 200 Kit (ThermoFisher Scientific) on Ion One Touch System. Sequencing was performed in BSRC 'Alexander Fleming' Genomics Facility using the Ion PI™ HiQ Sequencing 200 Kit and Ion Proton PI™ V2 chips (ThermoFisher Scientific) on an Ion Proton™ System, according to the manufacturer's instructions.

Analysis of mRNA-seq data

Mapping of sequencing reads to mm10 reference genome was performed as recommended by the manufacturer and gene differential expression analysis was performed using Bioconductor package DESeq through metaseqR pipeline, as described [56]. Quality control of the data was done by Multidimensional scaling analysis to confirm high correlation and reproducibility among samples replicates of each group. Downstream bioinformatics analysis was performed by generating volcano plots in R, using an in-house developed script and ggplot package. Functional Annotation tool from Genecodis was used as previously described [57] to analyze and find the top Biological Processes (BP) and Gene Ontologies (GO). Gene Set Enrichment Analysis (GSEA) (https://software.broadinstitute.org/gsea/index.jsp) was also conducted on all available MsigDb gene-expression signatures from the C5: GO BP category. FDR q-val and normalized enrichment score (NES) were utilized to determine the concordance differences between a given gene set and the ranked list of DE genes after Prox1 overexpression. RNA-seq raw data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE158904.

Fluorescence-activated cell sorting (FACS) analysis

Cultures of E14.5 embryonic mouse forebrain neurons were transfected using Amaxa electroporation with 6 µg of either *IRES-GFP* or *Prox1-IRES-GFP* constructs and further cultured for 2 days after electroporation. Cells were washed with DMEM with 1gr/L Glucose, resuspended in FACS buffer [Neurobasal medium (Invitrogen), 0.5% FBS and 1 mM EDTA; pH = 8], and filtered using a 50 µm filter (Falkon). Transgene expressing cells were sorted on BD FACSAria TM llu (BD biosciences). RNA extraction from isolated cells was performed using Nucleospin RNA XS, (Macherey Nagel).

Ca^{+2} imaging

For live Ca^{+2} imaging, 7-day-old E15.5 forebrain neurons, Amaxa electroporated with either *IRES-GFP* or *Prox1-IRES-GFP*, were loaded with Fura-2 AM, as described [58]. In particular, neurons were plated on poly-L-lysine-coated 48-well plates (10^5 cells/well), cultured for 7 days, incubated with 2 µM Fura-2 AM in culture medium for 30 min at 37 °C, and then returned to culture medium and incubated for 3 h at 37 °C. Consequently, medium was changed to a low K^+/Ca^{2+} buffer (129 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM glucose, 0.1% BSA, and 25 mM HEPES [pH 7.4]). Ca^{2+} measurements, before and after the addition of 50 mM KCl, to obtain the maximum Ca^{2+} response, were performed using a PTI live cell Imaging system (HORIBA Scientific) on a TE 2000U-inverted fluorescence microscope (Nikon, Osaka, Japan), with an intensified (CCD) camera (PTI-IC 200) and the Image Master software package, equipped with specific Fura-2 filters with emission wavelength 485–530 nm (Chroma Technology Corp, set 455–0053) and excitation wavelengths 350 ± 5 nm and 380 ± 5 nm. Cells were excited at 350 nm and 380 nm, and emission fluorescence images were acquired at 510 nm, every 10 s. Image sequences were analyzed using the FIJI software [59]. The Ca^{2+} response of each cell was determined using the region of interest (ROI) tool and the Multi-Plot function in FIJI, by measuring the Fura-2 fluorescence intensity at the two excitation wavelengths 350 nm and 380 nm, respectively.

Changes in Ca^{2+} were determined using temporal analyses on single cells that expressed the transgene (certified by GFP fluorescence) and the data were expressed as fluorescence ratios (350/380 ratio) as described [58]. Rmax% is the percentage of the peak (maximum) fluorescence ratio immediately after KCl addition (representing the peak response to KCl) relative to the average baseline fluorescence ratio before KCl addition.

Statistical analysis

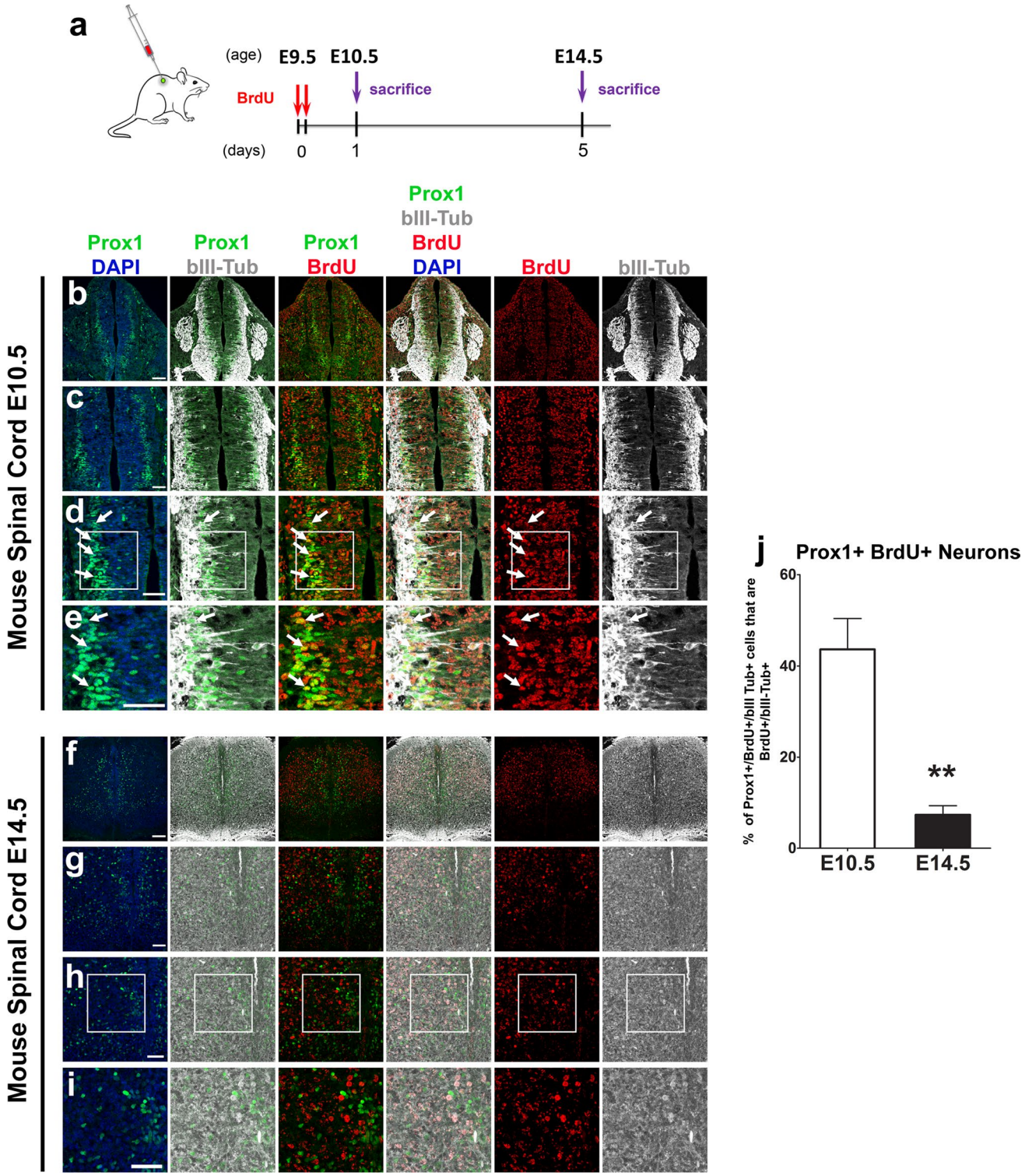
The measurements and experimental values from independent experiments were analyzed and compared by two-tailed Student's t test. The results are shown as mean values of independent experiments ± STDEV. p values < 0.05 were considered statistically significant.

All analyses were done using Microsoft Excel or GraphPad Prism 5 software.

Results

Prox1 endogenous expression is largely reduced during neuronal maturation in embryonic development

Although we and others have previously shown that Prox1 induces early neuronal identity [5–7, 39], it has not been investigated whether Prox1 regulates later differentiation processes. To tackle this question, we first examined whether



Prox1 endogenous expression is associated with neuronal maturation after acquisition of early post-mitotic, neuronal fate. To this end, we marked the newly born spinal cord neurons with two consecutive injections of BrdU (2-h interval) at the embryonic age E9.5 and then quantified the number of marked neurons (BrdU and bIII-Tubulin double-positive cells) that express Prox1 at an early (E10.5) and late (E14.5) developmental time-points (Fig. 1a). Interestingly, while Prox1 is expressed in about 43% of the marked, post-mitotic neurons (1 day after BrdU injections, E10.5) (Fig. 1b–e, j), this expression is highly down-regulated down to approximately 7% during transition towards more mature stages

◂**Fig. 1** Prox1 endogenous expression in early born neurons during development in mouse embryonic spinal cord. **a** Schematic drawing depicting the experimental procedure for BrdU injections (2 injections with 2-h interval) in E9 pregnant mice, which were then sacrificed after either 1 day (E10.5) or 5 days (E14.5). **b**–**e** Triple immunostainings of Prox1 (green), bIII-tubulin (gray), and BrdU (red) in E10.5 mouse spinal cord. c and d Larger magnifications of the images in b and c, respectively. e Larger magnification of the areas included in the square shapes of d. Arrows indicate representative neurons that co-express Prox1 and BrdU (Prox1 + /BrdU + /bIII-tubulin + cells). **f**–**i** Triple immunostainings of Prox1 (green), bIII-tubulin (gray), and BrdU (red) in E14.5 mouse spinal cord. **g**, **h** Larger magnifications of the images in f and g, respectively. i Larger magnification of the areas included in the square shapes of h. **j** Quantification of BrdU + neurons (bIII-tubulin +) that express Prox1 (% of Prox1 + cells that are BrdU + / bIII-tubulin + cells) in E10.5 and E14.5 mouse spinal cord ($n = 3$ mouse embryos, 5 sections per mouse embryos, $^{**}p < 0.01$). Scale bars: for (**b**, **f**) 100 µm; for (**c**–**e**, **g**–**i**) 50 µm

(5 days after BrdU injections, E14.5) (E10.5: 43.65 ± 6.776% of triple-positive cells *vs* E14.5: 7.333 ± 2% of triple-positive cells, $n = 3$ mouse embryos, 5 sections per mouse embryos, $p < 0.01$) (Fig. 1f–j). These observations suggest a negative correlation of Prox1 expression with neuronal maturation. Considering that axon elongation is initiated after acquisition of neuronal character, at the molecular level, these data raise the intriguing hypothesis that, although Prox1 acts as an initial inducer of neuronal identity in neuronal progenitors, it might suppress neurites and axon elongation in early born neurons, later during development. Therefore, Prox1 is initially expressed and then down-regulated in early born neurons to allow physiological extension of neurites and axons as well as neuronal maturation.

Prox1 suppresses neurite outgrowth in mouse and human neuroblastoma cells

To initially examine this hypothesis, we performed overexpression in mouse neuroblastoma cell line Neuro2A. In particular, we utilized a Tet-On-based overexpression system, capable to efficiently induce Prox1 or GFP expression (Fig. 2a-b). We have previously generated and used this system to study the effect of Prox1 on proliferation and cell cycle progression [30]. First, we confirmed the ability of these cells to overexpress Prox1 or GFP after doxycycline (Dox) administration with western blot and immunofluorescence analysis (Fig. 2a, b). Next, we showed that Prox1 overexpression was sufficient to inhibit neurite outgrowth in Neuro2A cells (GFP: 56.8 ± 3.107 µm *vs* Prox1: 34.2 ± 1.721 µm, $n = 3$ independent cultures, in total 160 neurites for GFP cells and 111 neurites for Prox1, $p < 0.01$) (Fig. 2c, e). Most importantly, Prox1 was able to block the retinoic acid (RA)-mediated induction of neurite extension in these cells (GFP: 110.3 ± 5.897 µm *vs* Prox1: 46.53 ± 1.938 µm, $n = 3$ independent cultures, in total 173 neurites for GFP cells and 130 neurites for Prox1, $p < 0.001$) (Fig. 2d, e). In addition, Prox1 suppresses neurite outgrowth in human neuroblastoma cell line SH-SY5Y in the presence of RA (GFP: 89.43 ± 5.576 µm *vs* Prox1: 39.07 ± 2.805 µm, $n = 3$ independent cultures, in total 88 neurites for GFP cells and 84 neurites for Prox1, $p < 0.01$) (Fig. 2f-g). Our observations demonstrate that Prox1 is sufficient for limiting neurite elongation of neuroblastoma cells.

Prox1 regulates numerous genes and pathways involved in neurite outgrowth and neuronal maturation

Considering that Prox1 is a transcriptional regulator, we next investigated the Prox1-mediated changes in gene expression that could drive repression of neurite outgrowth and/or neuronal maturation in neuroblastoma cells. mRNA-seq libraries were prepared from the Tet-On Neuro2A Prox1 and GFP overexpressing cells after 48 h of Dox administration, in the absence of RA. Out of 1860 genes that were differentially expressed (DE), 1006 were down-regulated and 854 up-regulated (Fig. 3a and Supplementary Table S1). Although Prox1 is traditionally associated with gene repression, we were not surprised to find a significant number of DE genes to be up-regulated, as indirect effects are also detected with this assay. By carrying out Gene Ontology (GO) analyses in down-regulated genes, we determined which biological processes were associated with Prox1 overexpression in neuroblastoma cells. Therefore, we demonstrated that many biological processes and molecular functions, associated with neurite outgrowth and neuronal maturation, are enriched in our datasets including nervous system development, neuron projection development, cell differentiation, axon guidance, neuron differentiation, learning or memory, neuron migration, and calcium ion-regulated exocytosis of neurotransmitter (Fig. 3b, highlighted in blue). Furthermore, gene set enrichment analysis (GSEA) in all differentially expressed genes revealed a suppressive effect of Prox1 on genes controlling cellular processes including cell projection, neuron projection, and neuron development (Supplementary Figure S1a-c).

Interestingly, we were able to identify a large number of individual genes, which were down-regulated by Prox1 and positively correlated with these biological processes and pathways. From this list, we focused on 12 genes, which have been previously highly associated with neurite and axon elongation as well as neuronal maturation. These included calcium homeostasis-related genes (*Camk2b*, *Camkk2*, *Camk4*, *Cacnb1,* and *Cacnb3*), retinoic acid receptor alpha (*Rara*), neuronal nitric oxide synthase 1 (*Nos1*), cyclin-dependent kinase 5 (*Cdk5*) genes as well as voltage-gated sodium channel genes (*Scn1b*, *Scn3b*, and *Scn9a*), and neuronal activity regulated gene (*Arc*) (Fig. 3c) [2, 60–81]. To further validate our RNA-Seq analysis and our selection

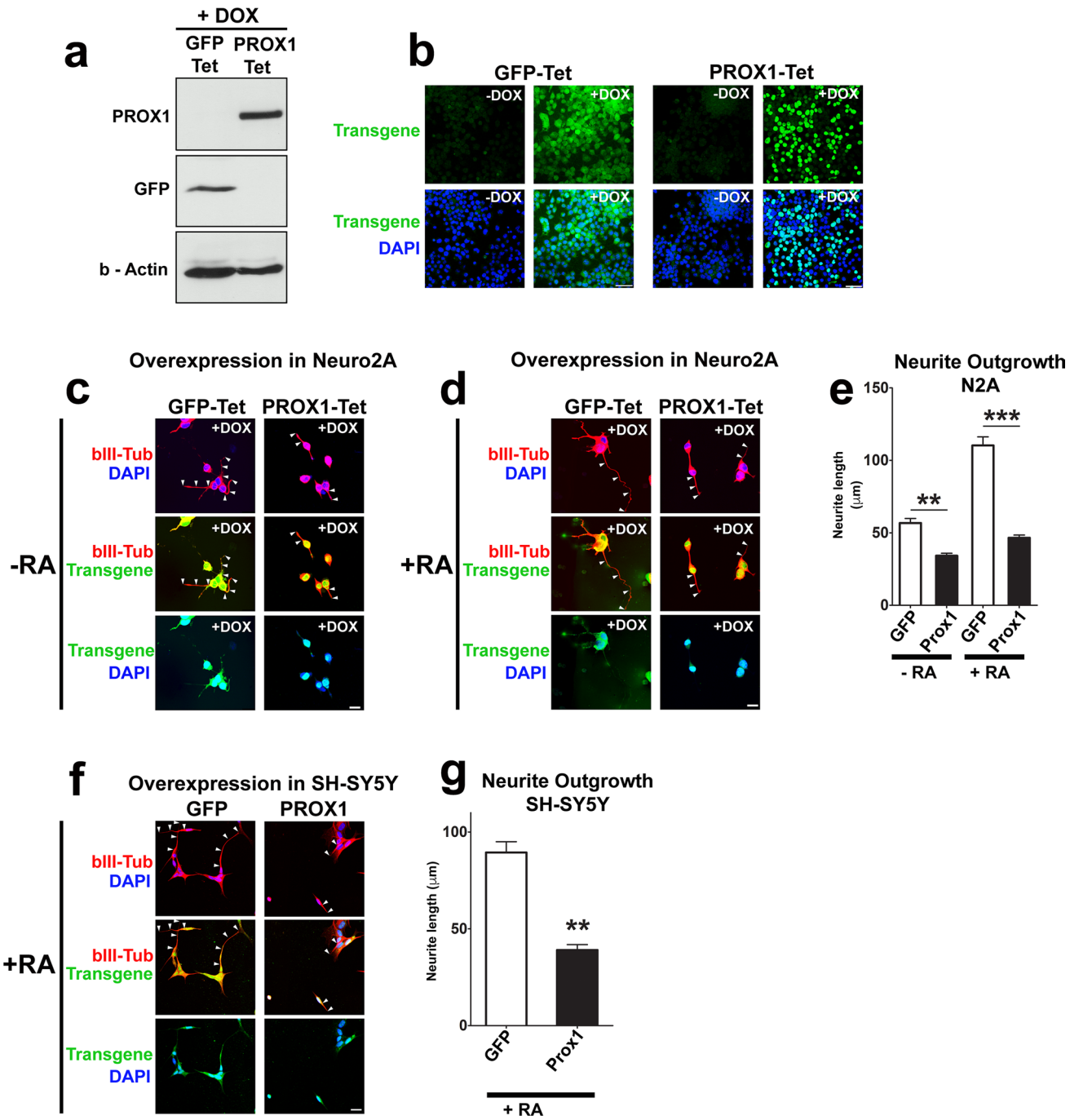
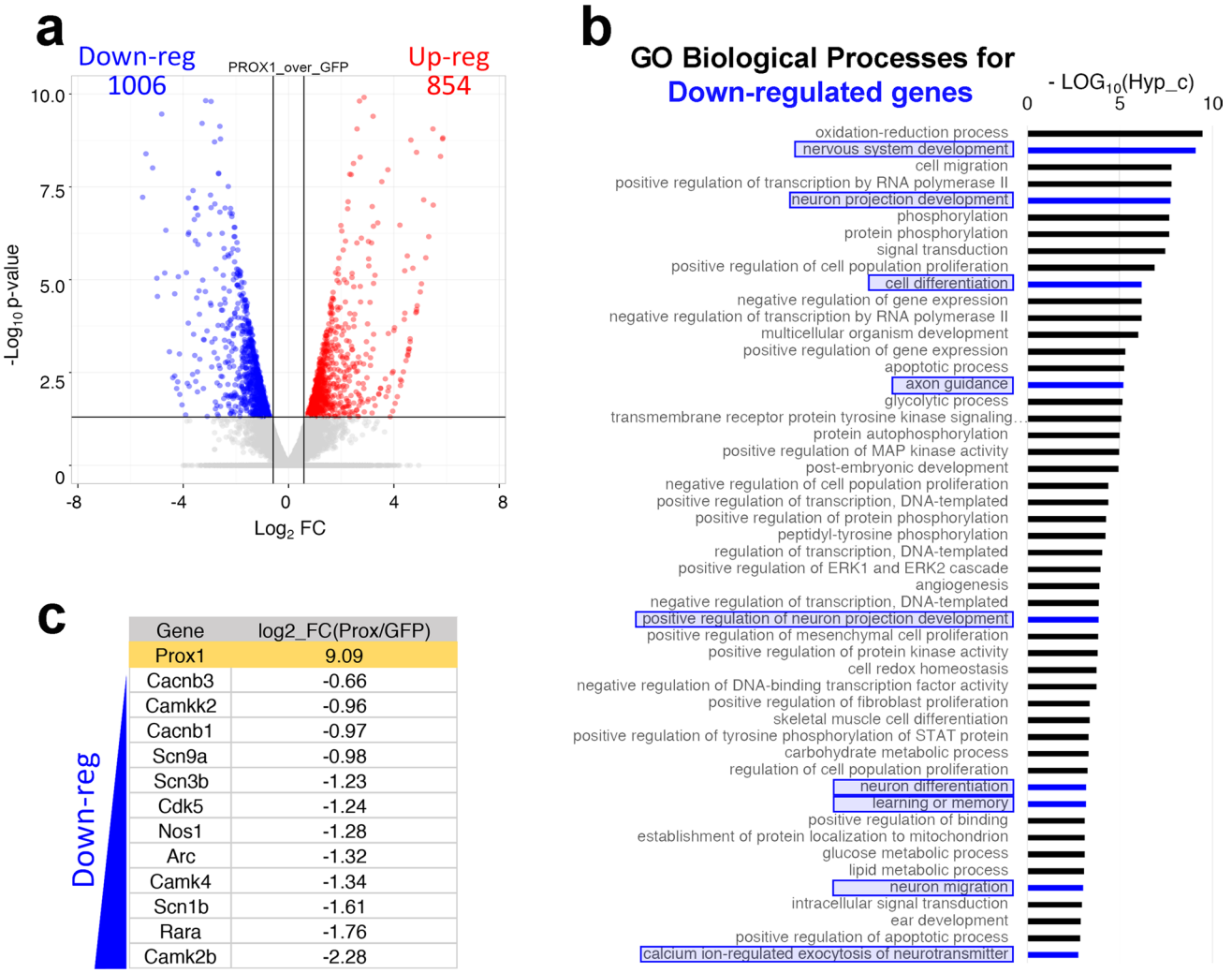


Fig. 2 Overexpression experiments indicate a Prox1-dependent inhibition of neurite outgrowth in mouse and human neuroblastoma cells. **a** Western blot analysis for Prox1, GFP, and actin of protein extracts from GFP-Tet and Prox1-Tet Neuro2A cells in the presence of Dox. **b** Immunostainings of GFP-Tet and Prox1-Tet Neuro2A cells with GFP and Prox1 (green), respectively, in the absence or presence of Dox demonstrate an overexpression of both transgenes in these cells in the presence of Dox. **c**, **d** Immunostainings of GFP-Tet or Prox1-Tet Neuro2A cells with bIII-Tubulin in the presence of Dox, either without (**c**) or with (**d**) RA. **e** Quantification of neurite length in Neuro2A cells, as described in (**c**) and (**d**) ($n=3$ independent cultures, $^{**}p<0.01$, $^{***}p<0.001$). **f** Immunostainings of GFP and Prox1-overexpressing SH-SY5Y cells with bIII-tubulin, in the presence of RA. **g** Quantification of neurite length, in GFP and Prox1-overexpressing SH-SY5Y, in the presence of RA ($n=3$ independent cultures, $^{**}p<0.01$). Arrowheads point to the longest neurite per cell, indicating its length. Scale bars: for (**b**) 50 μm; for (**c**, **d**) 20 μm; for (**f**) 25 μm



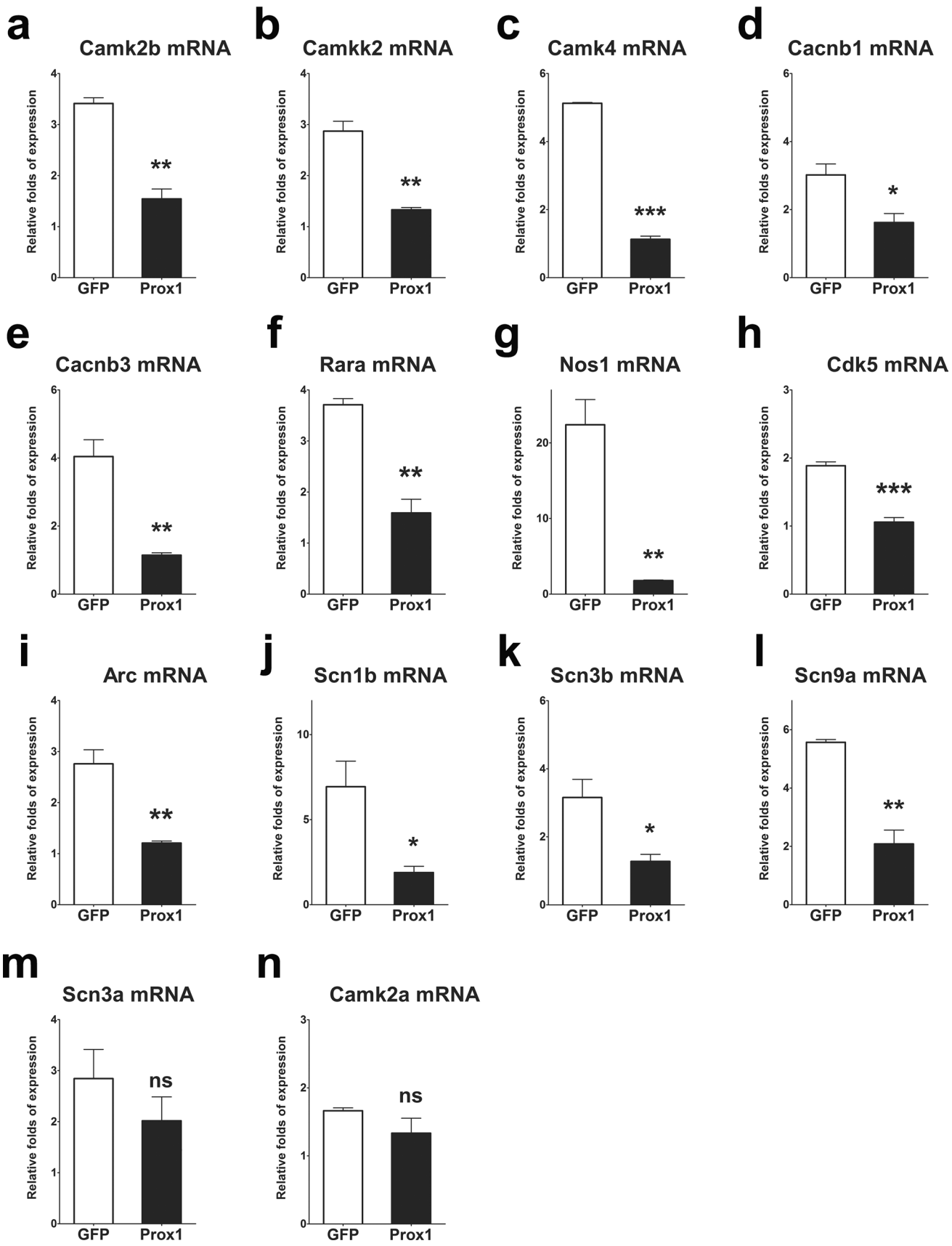
Gene	log2_FC(Prox/GFP)
Prox1	9.09
Cacnb3	-0.66
Camkk2	-0.96
Cacnb1	-0.97
Scn9a	-0.98
Scn3b	-1.23
Cdk5	-1.24
Nos1	-1.28
Arc	-1.32
Camk4	-1.34
Scn1b	-1.61
Rara	-1.76
Camk2b	-2.28

Fig. 3 Overexpression of Prox1 inhibits pathways involved in neurite outgrowth and neuronal maturation. **a** Volcano plot displaying gene expression changes between control (GFP) and Prox1-overexpressing Neuro2A cells analyzed by RNA-Seq. Differentially Expressed (DE) genes (up- and down-regulated upon Prox1 overexpression) are indicated in red and blue, respectively. An absolute Fold Change (FC) cut-off value of 0.58 in Log_2 scale and cut-off value of 1.3 for $-Log_{10}$ (p-val) were utilized as thresholds to identify DE genes (cut-offs represented as black thin lines on the graph). **b** Top 50 Biological Processes (Gene Ontology) terms associated with down-regulated genes. Most relevant terms of this study are highlighted. **c** Fold changes (FC) for selected genes, further analyzed in this study, are indicated

of genes, we performed real-time RT-qPCR assays of Prox1-overexpressing Neuro2A cells, as compared to GFP cells, in the absence of RA (Fig. 4a-l). Increased Prox1 levels were sufficient to significantly supress the expression of all these genes in our Neuro2A model (for *Camk2b, Camkk2, Rara, Nos1, Arc, Scn9a:* $p < 0.01$, $n = 3$ independent cultures; for *Cacnb3*: $p < 0.01$, $n = 4$ independent cultures; for *Cacnb1, Scn1b, Scn3b*: $p < 0.05$, $n = 3$ independent cultures, for *Camk4, Cdk5*: $p < 0.001$, $n = 3$ independent cultures). To evaluate the specificity of our results, we also examined the effect of Prox1 on *Camk2a* gene [not significant (n.s.), $p > 0.05$, $n = 3$ independent cultures], another key player in calcium signaling [82, 83], and *Scn3a* gene (n.s., $p > 0.05$, $n = 3$ independent cultures), and another voltage-gated sodium channel, which according to our RNA-seq analysis, was not differentially expressed (Supplementary Table S1). Interestingly, no significant differences were observed (Fig. 4m, n), indicative of a specific action of Prox1 on the 12 selected genes.

Prox1 inhibits neurite elongation in CNS neurons

To explore whether the negative effect of Prox1 on neurite outgrowth and gene expression program is recapitulated in a more physiological situation, we employed an ex vivo culturing system using primary neurons isolated from embryonic mouse forebrain (E15.5) and spinal cord (E14.5). Consistent with our observations in neuroblastoma cells,


a
Camk2b mRNA
Relative folds of expression
**
GFP
Prox1
b
Camkk2 mRNA
Relative folds of expression
**
GFP
Prox1
c
Camk4 mRNA
Relative folds of expression
***
GFP
Prox1
d
Cacnb1 mRNA
Relative folds of expression
*
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Prox1
e
Cacnb3 mRNA
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**
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Prox1
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Rara mRNA
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Nos1 mRNA
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Cdk5 mRNA
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n
Camk2a mRNA
Relative folds of expression
ns
GFP
Prox1

◂**Fig. 4** Prox1 suppresses the expression of genes correlated with neurite outgrowth and neuronal maturation in Neuro2A cells. **a–l** Real-time RT-qPCR analysis showing the expression levels of *Camk2b* (**a**), *Camkk2* (**b**), *Camk4* (**c**), *Cacnb1* (**d**), *Cacnb3* (**e**), *Rara* (**f**), *Nos1* (**g**), *Cdk5* (**h**), *Arc* (**i**), *Scn1b* (**j**), *Scnb3* (**k**), and *Scn9a* (**l**) in GFP or Prox1-overexpressing Neuro2A cells. **m–n** Real-time RT-qPCR analysis showing no significant differences in the expression levels of *Scn3a* (m) and *Camk2a* (n), in GFP or Prox1-overexpressing Neuro2A cells. In all cases, $n=3$ independent cultures, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, ns: $p>0.05$

Prox1 was capable to inhibit the outgrowth of the longest bIII-Tub + process per neuron, in mouse forebrain neurons (GFP: 111.5 ± 11.01 µm *vs* Prox1: 62.7 ± 10.89 µm, $n=4$ independent cultures, in total 212 neurites for GFP cells and 287 neurites for Prox1, $p<0.05$) (Fig. 5a, b). To further confirm the reduction in neurite length, we performed the same experiments by immunostaining the neuronal cultures with Tau, an axonal marker (GFP: 118 ± 2.005 µm *vs* Prox1: 83.33 ± 5.160 µm, $n=3$ independent cultures, in total 286 neurites for GFP cells and 262 neurites for Prox1, $p<0.01$) (Supplementary Figure S2). This reduction in differentiation potential is further illustrated by plotting the distribution of neurons against the length of their neurites (Fig. 5c). In agreement with these data and the expression studies in Fig. 1, Prox1 was also sufficient to strongly suppress neurite extension in mouse spinal cord neurons, as shown by measuring the longest bIII-Tub + process per neuron (GFP: 90.92 ± 6.518 µm *vs* Prox1: 57.34 ± 3.806 µm, $n=5$ independent cultures, in total 166 neurites for GFP cells and 186 neurites for Prox1, $p<0.01$) (Fig. 6a-c) or Tau positive neurites (GFP: 117.8 ± 6.018 µm *vs* Prox1: 51.27 ± 8.907 µm, $n=3$ independent cultures, in total 72 neurites for GFP cells and 55 neurites for Prox1, $p<0.01$) (Supplementary Figure S3).

To delineate the in vivo role of Prox1 in neurite elongation during embryonic development, we performed in utero electroporation experiments (IUE). To this end, we injected Prox1-overexpressing or control plasmid (empty vector) in the developing cortex of E14.5 mouse embryos. In both conditions, we co-injected GFP expressing plasmid to track the electroporated neurons (Tau +) and their neurites three days later (E17.5) (Fig. 7). Remarkably, and in agreement with our previous ex vivo experiments, there was a significant decrease in the length of neurites of GFP + /Tau + neurons in the Prox1-overexpressing embryos as compared to the control embryos (empty vector + GFP: 77.73 ± 4.585 µm *vs* Prox1 + GFP: 35.70 ± 2.558 µm, $n=3$ embryos, in total 92 neurites for GFP embryos and 139 neurites for Prox1 embryos, $p<0.01$) (Fig. 7).

Furthermore, to address whether Prox1 is necessary to inhibit neurite outgrowth in primary neuronal cells, we performed shRNA-mediated knock-down of Prox1 by using AMAXA electroporation in neuronal cultures from ventral forebrain of E14.5 embryo. We chose to focus on this brain area and embryonic day due to the fact that Prox1 expression is sustained in differentiated neurons from ventral forebrain during their transition from progenitor phase to early mature neuronal phase. In the majority of other CNS areas, Prox1 is only transiently expressed [4–7, 35, 40], and therefore, we cannot efficiently follow the Prox1 knock-down in ex vivo cultures. We confirmed here that endogenous Prox1 expression is sustained in the context of ex vivo neuronal cultures from E14.5 ventral forebrain (data not shown and Fig. 8a). We then demonstrated that shRNA against Prox1 down-regulates endogenous Prox1 expression in these neuronal cultures (Fig. 8a). More importantly, shProx1-GFP led to a significant induction in the neurite length of Tau + ventral forebrain neurons as compared to control vector, shSCR-GFP (shSCR-GFP: 47.45 ± 2.832 µm *vs* shProx1-GFP: 60.83 ± 3.369 µm, $n=4$ independent cultures, in total 304 neurites for shSCR-GFP cells and 255 neurites for shProx1-GFP, $p<0.05$) (Fig. 8b, c).

We next tested whether the Prox1-regulated genes, identified from our inducible Neuro2A system, are also transcriptionally repressed by Prox1 in primary mouse CNS neurons (E14.5). To this end, we first performed Amaxa electroporation to overexpress Prox1-IRES-GFP or IRES-GFP alone, as a control, in ex vivo cultured forebrain neurons. Then, we used FACS to isolate the GFP expressing cells (Fig. 9a). RNA was subsequently extracted from these cells and analyzed by real-time RT-qPCR assay. Consequently, we were able to verify the negative action of Prox1 in most of the genes identified in Neuro2A system (Fig. 9b-h). Interestingly, the same five genes, involved in calcium signaling pathway, which were identified in Neuro2A cells, were significantly reduced in primary neurons. In particular, genes encoding for intracellular calcium signaling mediators (*Camk2b*, *Camkk2,* and *Camk4*), two calcium channel subunits (*Cacnb1* and *Cacnb3*) as well as *Rara* and *Nos1* genes were strongly affected (Fig. 9b-h) (for *Camk2b*: $p<0.01$, $n=4$ independent cultures; for *Camkk2, Camk4, Rara*: $p<0.05$, $n=3$ independent cultures; for *Cacnb1*: $p<0.05$, $n=5$ independent cultures, for *Cacnb3*: $p<0.05$, $n=2$ independent cultures; for *Nos1*: $p<0.001$, $n=2$ independent cultures). Rara facilitates the inductive role of retinoic acid signaling in neuritogenesis [66, 73, 84, 85], while Nos1 has been reported to promote neurite elongation and facilitate retinoic acid signaling-mediated effects on neuritogenesis [67, 74, 75, 86]. Most importantly, Prox1 overexpression exerted a negative effect on *Camk2b* gene expression, which is a major positive regulator for the initial steps of neurite extension and axon elongation [2, 60, 76, 77, 87]. On the contrary, Prox1 did not affect *Camk2a* gene, another calcium signaling modulator [82, 83], or other genes related to neuronal maturation, including *Cdk5* and *Arc* [68, 70, 71] (Fig. 9i-k) [for all three genes: $p>0.05$

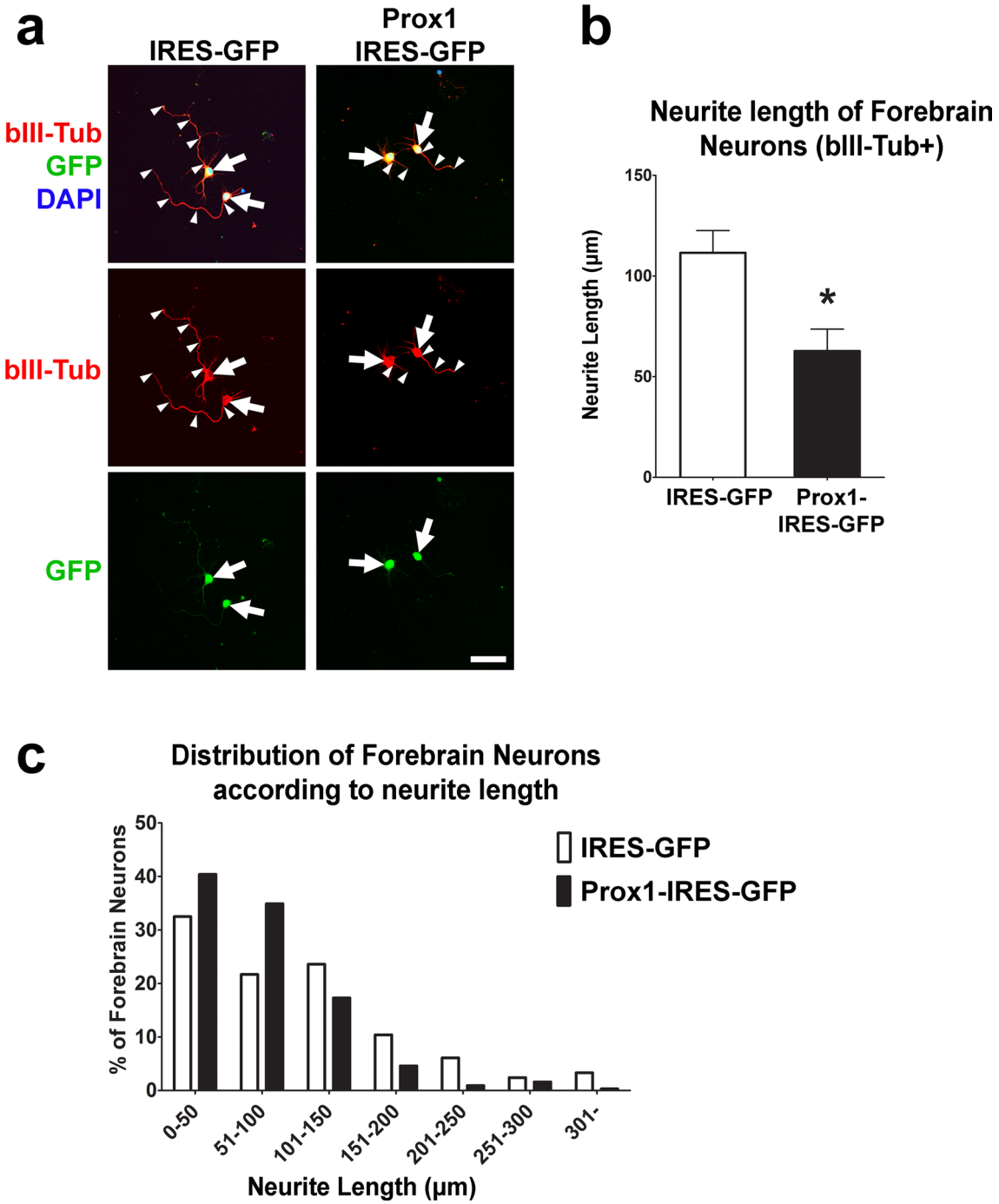


Fig. 5 Prox1 inhibits neurite elongation in mouse embryonic forebrain neurons. **a** Double immunostainings of forebrain neurons isolated from E15.5 mouse and electroporated with IRES-GFP or Prox1-IRES-GFP, with bIII-tubulin (red) and GFP (green). The arrows indicate neurons that co-express the transgene (IRES-GFP or Prox1-IRES-GFP) and bIII-tubulin. Arrowheads point to the longest bIII-Tub + process, representing the neurite length. **b** Quantification of neurite length in IRES-GFP and Prox1-IRES-GFP neurons, $n = 4$ independent cultures, $^{*}p < 0.05$. **c** Distribution of neurons electroporated with GFP-IRES and Prox1-IRES-GFP according to neurite length. Scale bar: 50 μm

(n.s.), $n = 3$ independent cultures]. Interestingly, *Cdk5* and *Arc* are significantly down-regulated by Prox1 in the cellular context of Neuro2A cells, suggesting Prox1-specific effects on gene expression, probably due to the existence of different interacting partners for Prox1 or differences in the chromatin microenvironment of these genes. Moreover, Prox1 represses *Cacnb1* and *Cacnb3* genes, encoding beta subunits of voltage-gated calcium channels (VGCCs), which

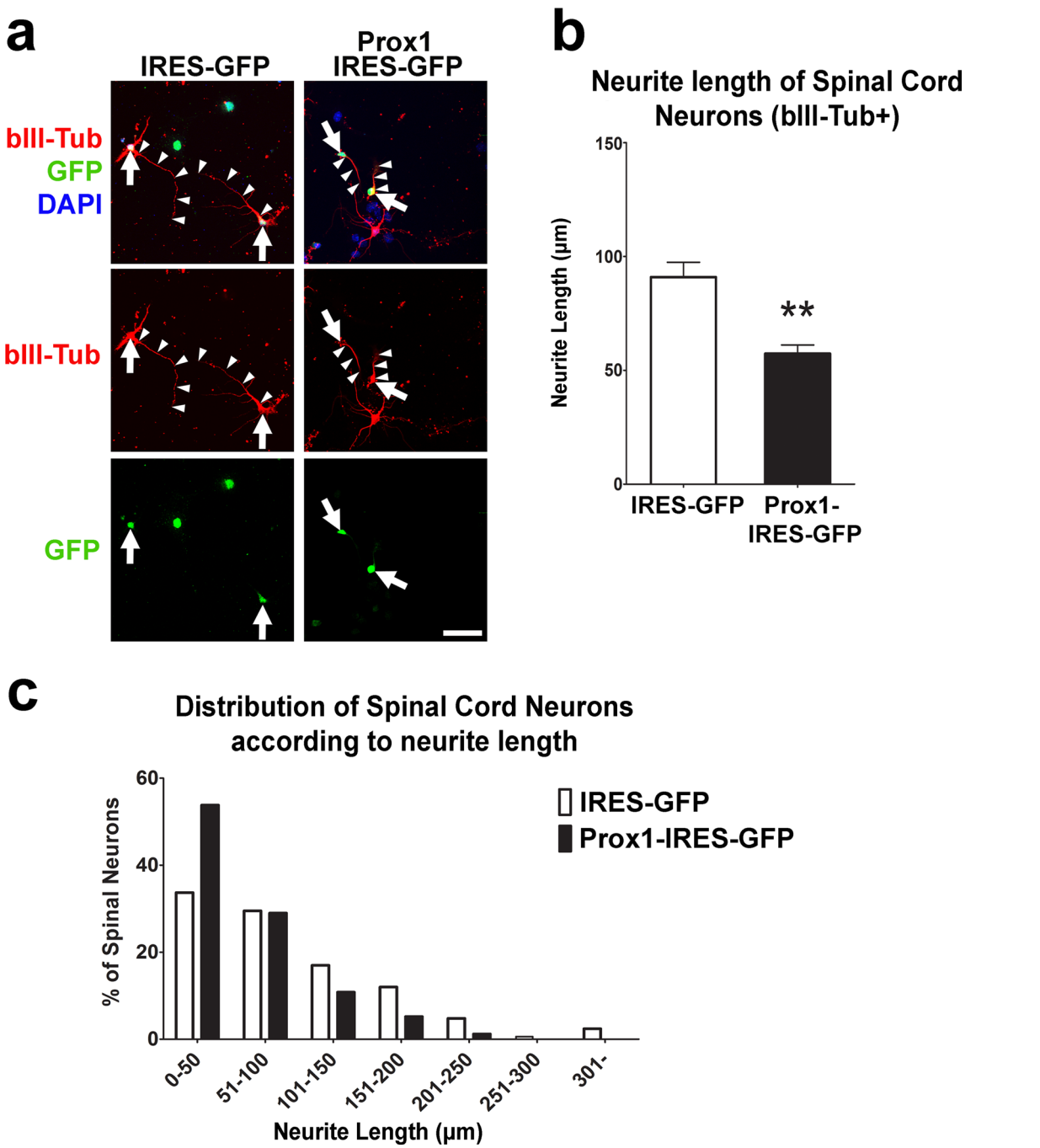


Fig. 6 Prox1 inhibits neurite elongation in mouse embryonic spinal cord neurons. **a** Double immunostainings of spinal cord neurons isolated from E14.5 mouse and electroporated with IRES-GFP or Prox1-IRES-GFP, with bIII-Tubulin (red) and GFP (green). The arrows indicate neurons that co-express the transgene (IRES-GFP or Prox1-IRES-GFP) and bIII-Tubulin. Arrowheads point to the longest bIII-Tub+process, representing the neurite length. **b** Quantification of neurite length in IRES-GFP and Prox1-IRES-GFP neurons, $n=5$ independent cultures, $^{**}p<0.01$. **c** Distribution of neurons electroporated with GFP-IRES and Prox1-IRES-GFP according to neurite length. Scale bar: 50 µm

participate in extracellular calcium entry in neuronal cells [63, 64]. Increased intracellular levels of Ca^{2+} activate calcium/calmodulin-dependent protein kinases to promote neurite outgrowth and neuronal maturation [44, 45]. Moreover, VGCCs have been related to neurite outgrowth [65]. These data imply a specific role of Prox1 in suppressing critical components of calcium entry and signaling to inhibit extension of neuronal processes.

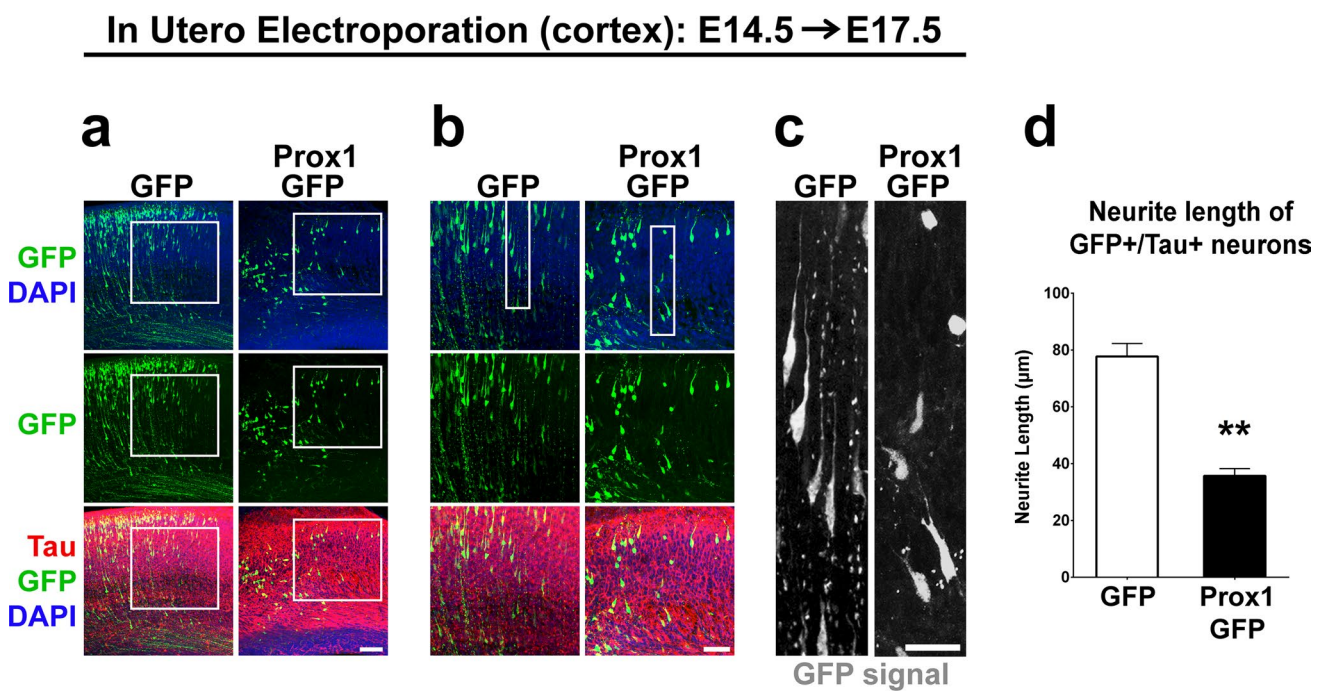


Fig. 7 In vivo Prox1 overexpression by in utero electroporation suppresses neurite elongation in the developing mouse cortex. **a** Double immunostainings with GFP (green) and Tau (red) of sections from E17.5 mouse brains that were in utero electroporated with either GFP or GFP together with Prox1. Cell nuclei were stained with DAPI (blue). **b** Micrographs represent larger magnification of the areas included into the square shapes of a. **c** Micrographs (GFP signal only) represent larger magnification of the areas included in the rectangular shapes of b. Note the clear difference in the length of neurites between the two conditions. **d** Quantification of neurite length in GFP and GFP + Prox1 neurons, $n = 3$ embryos, $^{**}p < 0.01$ Scale bars: for (**a**) 75 μm; for (**b**) 50 μm; for (**c**) 25 μm

Prox1 overexpression impairs Ca^{2+} entry and CREB phosphorylation in neuronal cells

To examine this hypothesis, we tested whether Prox1 overexpression has an impact on stimuli-driven Ca^{2+} responses. Accordingly, we cultured Prox1-IRES-GFP or IRES-GFP electroporated neurons for 7 days and then measured the Ca^{2+} influx after stimulation with KCl with the use of Fura-2 AM Dye. The electroporated cells were followed by the GFP signal, which we confirmed that, under our specific experimental conditions, was not significantly interfered with Fura-2 signal (Supplementary Figure S4). Forebrain neurons overexpressing Prox1 exhibited a significantly lower Ca^{2+} entry upon KCl-mediated depolarization as compared to control cells (GFP: $284.6 \pm 16.84\%$Rmax *vs* Prox1: $196.5 \pm 10.17\%$Rmax, $n = 70$ cells, $p < 0.001$) (Fig. 10a-d). Since intracellular levels of Ca^{2+} act as an important messenger, via Ca^{2+}/calmodulin-dependent protein kinases, to induce phosphorylation of CREB (cAMP-response element-binding protein) [77, 88, 89], we tested whether Prox1 could influence CREB phosphorylation in primary neurons. First, we confirmed that, under our experimental conditions, CREB phosphorylation was induced in neuronal cells after KCl addition (Supplementary Figure S5). Then, we observed a significant reduction in CREB phosphorylation after KCl induction in Prox1-overexpressing neurons as compared to GFP (GFP: $82.13 \pm 1.924\%$ vs Prox1: $59.25 \pm 3.964\%$, $n = 4$ independent cultures, $p < 0.01$) (Fig. 10e, f). These data are in agreement with the Prox1 effect on calcium entry and genes associated with calcium signaling.

Finally, to investigate whether the effect of Prox1 on neurite elongation is mediated through calcium signaling, we utilized a constitutively active form of CREB (VP16-CREB fusion protein construct) in rescue experiments. To this end, overexpression of VP16-CREB is able to partially reverse the inhibitory effect of Prox1 on neurite outgrowth in forebrain neurons (GFP: 96.33 ± 1.121 μm; Prox1: 57.58 ± 4.799 μm; VP16-CREB + Prox1: 85.75 ± 1.258 μm, $n = 3$ independent cultures, in total 227 neurites for GFP cells, 194 neurites for Prox1 and 187 neurites for VP16-CREB + Prox1; for GFP vs Prox1 $p < 0.01$; for Prox1 vs VP16-CREB + Prox1 $p < 0.01$; for GFP vs VP16-CREB + Prox1, $p < 0.01$) (Supplementary Figure S6). The observation that this rescue is partial, is probably due to the ability of Prox1 to affect many other genes and pathways involved in neurite and axon outgrowth, as documented in Fig. 3.

Collectively, our results propose a regulatory role of Prox1 in calcium homeostasis and signaling through which, at least partially, Prox1 affects neurite elongation and neuronal maturation during the differentiation.

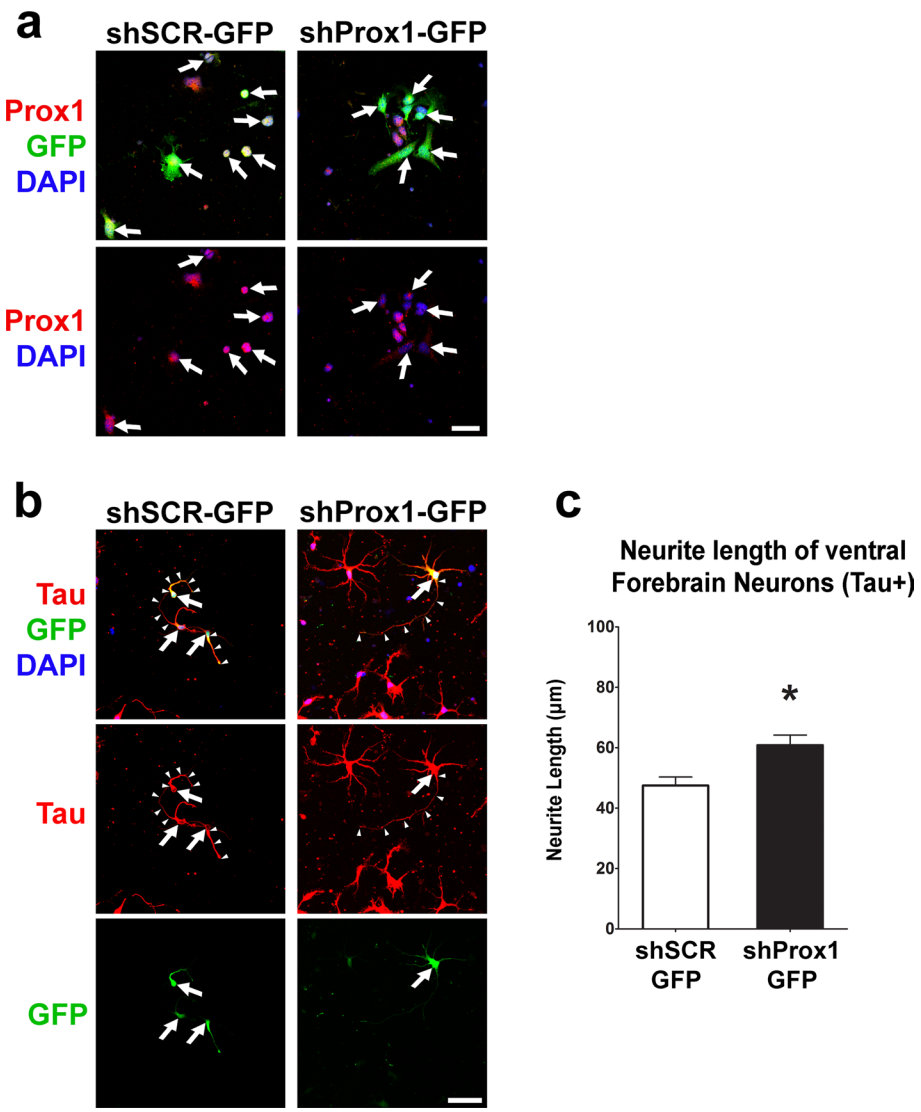
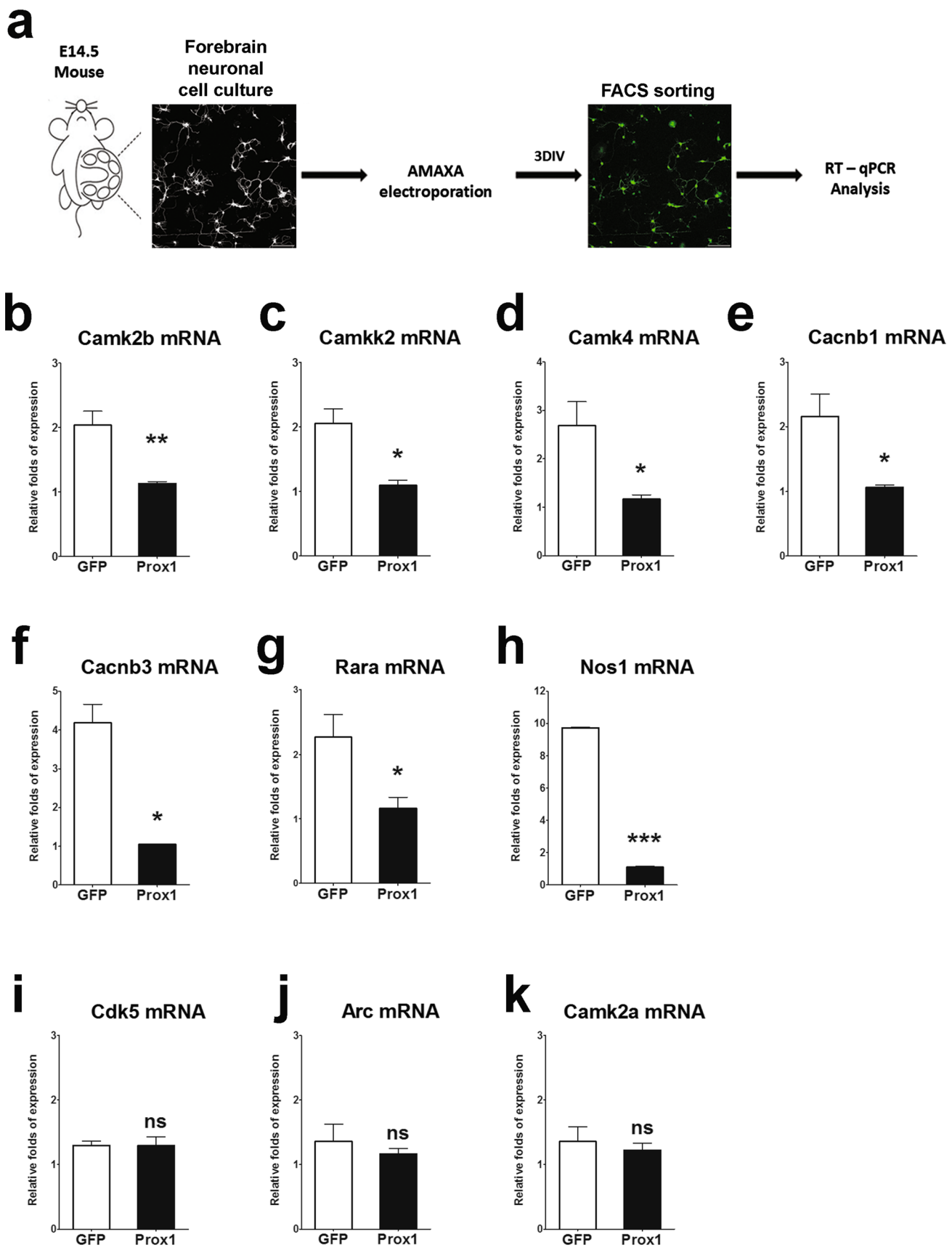


Fig. 8 shRNA-mediated knockdown of Prox1 promotes neurite outgrowth in ventral forebrain neurons from E14.5 mouse embryo. **a** Double immunostainings of ventral forebrain neurons, electroporated with shSCR-GFP or shProx1-GFP, with Prox1 (red) and GFP (green). The arrows indicate cells that express the GFP transgene. Note the down-regulation of Prox1 expression in the shProx1-GFP electroporated neurons but not in the shSCR-GFP neurons. **b** Double immunostainings of the cells from the same experimental conditions with Tau (red) and GFP (green). The arrows indicate neurons that co-express the GFP and Tau. Arrowheads point to the Tau + neurites, representing the neurite length. **c** Quantification of the neurite length in shSCR-GFP and shProx1-GFP neurons, $n = 4$ independent cultures, $^*p < 0.05$. Scale bars: for (**a**) 25 µm; for (**b**) 50 µm

Discussion

In the CNS, during early developmental stages, Prox1 expression is observed in intermediate neuronal progenitor cells in the ventricular zone (VZ) and subventricular zone (SVZ). In these cells, Prox1 regulates the balance between proliferation and differentiation decisions. Prox1 actively directs exit of the cell cycle in progenitor cells and induces neuronal differentiation [5, 6]. Interestingly, in the majority of neuronal populations, Prox1 expression is then down-regulated during progression of the newly-born neurons towards a mature fully-differentiated phenotype [4–7, 35, 36, 38–40]. Here, we show that endogenous Prox1 expression is dramatically reduced in bIII-Tubulin-positive neurons (experimentally marked with BrdU) during transition from developmental stage E10.5 to E14.5 in the mouse spinal cord. These findings raised the hypothesis that Prox1 may exert a regulatory role in suppressing neuronal maturation. In agreement, overexpression in neuroblastoma cell lines demonstrated that Prox1 is sufficient to inhibit neurite extension in these specific cells. Consistently, further analysis in mouse primary neuronal cultures from embryonic forebrain and spinal cord as well as in *vivo* in mouse embryonic brain revealed a negative effect of Prox1 on neurite elongation. These data suggest that Prox1 is a unique transcriptional regulator, able to mediate a dual function of initially promoting cell cycle exit and neuronal differentiation of neural progenitor cells [5, 6], and then suppressing neurite outgrowth in nascent neurons (this study). We speculate that this action may provide the appropriate time window for the nascent neurons to find the correct place or migratory route in the developing CNS environment, prior to initiation of mature neurite extension and axon elongation.

However, in specific CNS areas, Prox1 expression is selectively maintained in the post-mitotic, mature neurons of the adult brain, including granule cells of the dentate


a
E14.5
Mouse
Forebrain
neuronal
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AMAXA
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3DIV
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Analysis
b
Camk2b mRNA
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Camkk2 mRNA
d
Camk4 mRNA
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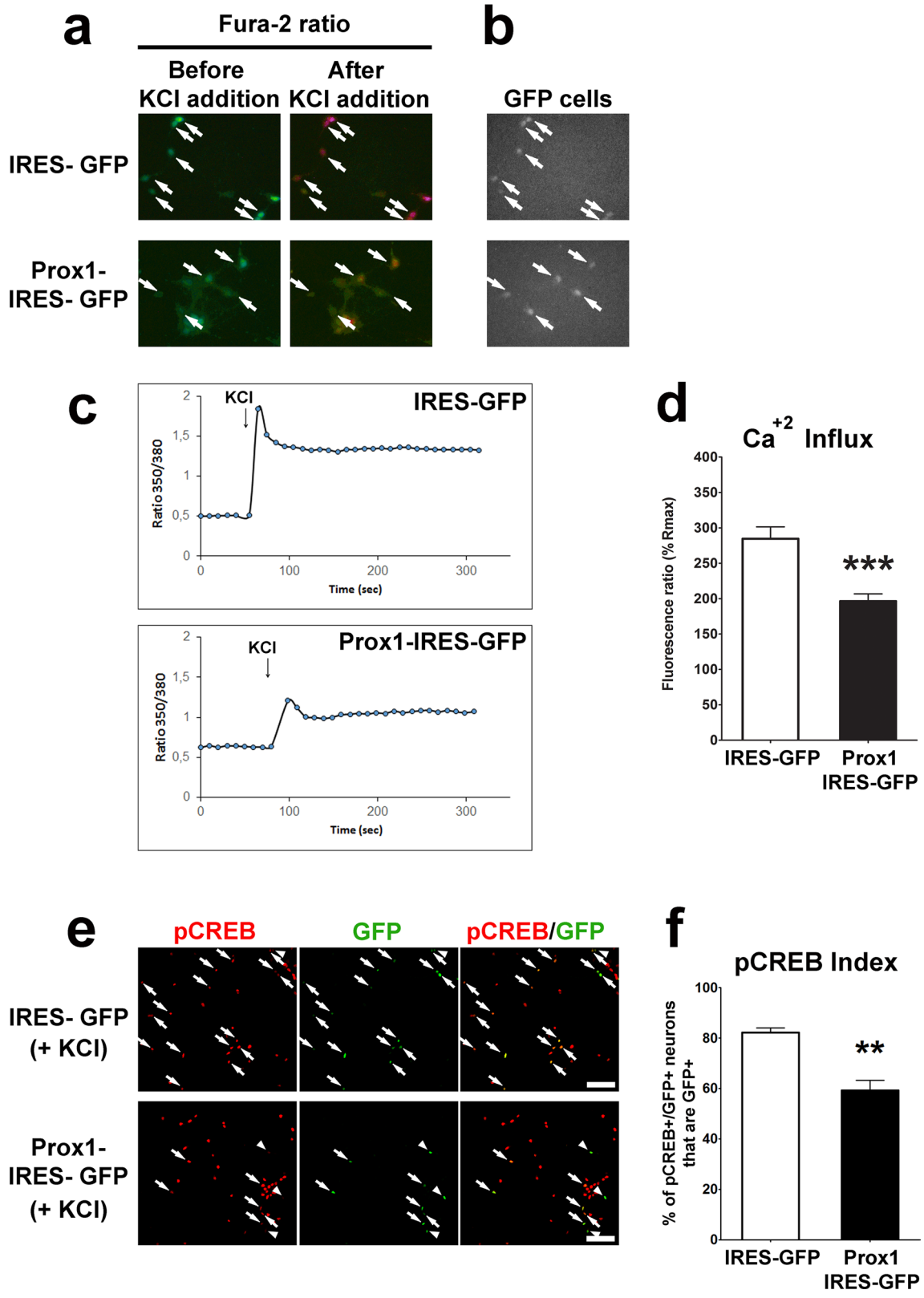
◂**Fig. 9** Prox1 suppresses the expression of genes correlated with neurite elongation and neuronal maturation in Neurons. **a** Experimental design for isolation of RNA derived from Amaxa electroporated E14.5 forebrain neurons expressing either IRES-GFP or Prox1-IRES-GFP. Transfected cells (GFP positive) were sorted by FACS. **b–h** Real-time RT-qPCR analysis showing the expression levels of *Camk2b* (**b**), *Camkk2* (**c**), *Camk4* (**d**), *Cacnb1* (**e**), *Cacnb3* (**f**), *Rara* (**g**), and *Nos1* (**h**) in GFP only or Prox1-overexpressing Neuro2A cells. **i–k** Real-time RT-qPCR analysis showing no significant differences in the expression levels of *Cdk5* (i), *Arc* (j), and *Camk2a* (k), in GFP only or Prox1-overexpressing Neuro2A cells. For (b), $n=4$ independent cultures; for (**c**, **d**, **g**), $n=3$ independent cultures; for (**e**), $n=5$ independent cultures; for (**f**, **h**), $n=2$ independent cultures; for (**i–k**), $n=3$ independent cultures. $*p<0.05$, $**p<0.01$, $***p<0.001$, ns: $p>0.05$

gyrus of hippocampus, CGE-derived GABAergic cortical interneurons as well as dispersed interneurons of striatum and amygdala [4, 35, 36, 40, 90]. In the lineage of CGE-derived cortical interneurons, Prox1 controls the genetic program for specification and migration [35, 36]. In the dentate gyrus of adult hippocampus, Prox1 expression is also maintained and functions as a post-mitotic cell fate regulator that is necessary for the differentiation, survival, and maturation of granule cells [37, 38, 90]. Therefore, the constitutive expression of Prox1 in these specific neuronal sub-types denotes a differential and distinct role of this transcriptional regulator as compared to its ability to suppress neurite elongation and neuronal maturation in other CNS areas, demonstrated in this study. The molecular mechanism by which a transcriptional regulator orchestrates multiple phenotypic outcomes may be explained by differential interactions with a large repertoire of binding partners and thus differential regulation of downstream target genes. In this scenario, different binding partners could direct the context-dependent actions of Prox1 in different neuronal sub-types or lineages. Consistently, Prox1 has been previously reported to interact with many transcription factors, where it acts as a co-repressor protein by recruiting chromatin modulators or other transcriptional regulators. For example, Prox1 interacts and controls the activity of Pax6, CoupTFII, Lrh1 (Nr5a2), Sf1 (Nr5a1), RORs, ERRa, Pgc-1a, HNF-4a, Klf2 as well as Hdac3, Lsd1, Crebbp (Cbp), and Ep300 (p300) [6, 19, 21, 24, 26, 50, 91–96]. Thus, Prox1 may be able to regulate many distinct phenotypes, cellular processes, or developmental events depending on its spatiotemporal expression pattern and its partners. In agreement, although an anti-proliferative and tumor suppressor function of Prox1 has been demonstrated in several tissues [12, 29, 30, 32–34, 97–100], an opposite oncogenic and pro-proliferative action has been proposed in other cell types and tissues [25, 27, 28, 101–105]. Collectively, these findings highlight the complexity of Prox1 molecular and functional interactions and are in good accordance with our observations suggesting context-dependent roles of Prox1 in neurite elongation and neuronal maturation. It would be interesting to further investigate which regulatory networks define these outcomes and how they are implemented in space and time.

To understand the Prox1 effect on neurite outgrowth and neuronal maturation at the transcription regulation level, we performed RNA-Seq in Neuro2A cells. In agreement with our phenotypic experiments in these cells, bioinformatic analysis of the RNA-Seq data revealed a number of gene sets and pathways that were down-regulated and associated with nervous system development, neuron projection development, axon guidance, learning or memory, neuron migration, calcium ion-regulated exocytosis of neurotransmitter. By confirming these observations in primary neurons, we documented a repressive action of Prox1 on the expression of many genes related to calcium signaling pathway. Intracellular Ca^{2+} functions as a secondary signal at the neuronal cells, having crucial roles in many aspects of neuronal development including neurite extension, axon elongation, and growth cone motility [46, 106–110]. Interestingly, we identified two genes encoding for beta subunits of L-type VGCCs as Prox1 downstream targets (*Cacnb1* and *Cacnb3*). L-type calcium channels allow a Ca^{2+} influx into the cell, and have been previously associated with several fundamental neuronal processes, such as neurite outgrowth, synaptic transmission, gene transcription, and cytoskeletal remodeling [64, 65, 111–113]. Knock-down or inhibition of VGCCs channels results in impaired calcium entry in neuronal cells and downstream calcium signaling [63–65].

Moreover, Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), including the Camk2b, Camkk2, and Camk4, were also found to be suppressed by Prox1. These kinases act as intermediate signaling molecules between increased intracellular calcium levels and neurite outgrowth [2], Therefore, Prox1-mediated down-regulation of CaMKs could explain its inhibitory effect on neurite elongation. In particular, Camk2b has been extensively associated with a positive action on neurite outgrowth and neuronal processes, such as neurotransmitter synthesis and release as well as synaptic plasticity, learning, and memory [60, 76, 77, 87]. Accordingly, pharmacological inhibition or knock-down of Camk2b heavily impairs the ability of mouse and human neuroblastoma cells as well as mouse primary neurons to extend neurites and axons, respectively [60, 76, 77]. In addition, the other two Prox1-repressed CaMKs, Camkk2 and Camk4, are also involved in these neuronal maturation events. Specifically, neurite and axon outgrowth in neuroblastoma cells and primary neurons were significantly decreased after pharmacological or genetic inhibition of CaMKKs [61, 78, 79, 114, 115]. Furthermore, Camk4 has been previously reported to promote neuritogenesis via phosphorylation of CREB [62, 80, 81, 89, 116]. Interestingly, we also show that Prox1 reduces both Camk4 expression and CREB phosphorylation. This regulatory action could also be responsible, in



part, for the Prox1 phenotypic effect. However, based on our observations, we cannot exclude the possibility of an indirect regulation of these genes by Prox1, and thus, the detailed molecular mechanism should be further investigated. Taken together, these results propose that Prox1 acts on different components of calcium signaling cascade, either directly or

◂**Fig. 10** Prox1 is sufficient to reduce the Ca^{+2} response and CREB phosphorylation after stimulation with KCl, in primary mouse forebrain neurons. **a**, **b** Representative images demonstrating the 350/380 fluorescence ratio after loading GFP-IRES or Prox1-IRES-GFP neurons with Fura-2 AM dye, before and after stimulation with KCl (**a**) and corresponding images showing the GFP expression (**b**). The arrows indicate GFP-IRES or Prox1-IRES-GFP neurons that express GFP. **c** Representative Ca^{+2} recordings depicting changes in 350/380 fluorescence ratio of Fura-2, after KCl application, as indicated. The arrows denote the time of KCl addition. **(d)** Quantitative analysis of the percentage of fluorescence ratio, normalized to baseline fluorescence ($n=70$ cells). **e** Double immunostainings of KCl-mediated depolarized forebrain neurons isolated from E15.5 mouse and electroporated with either IRES-GFP or Prox1- IRES- GFP, with pCREB (red) and GFP (green). The arrows indicate neurons that co-express the transgene (IRES-GFP or Prox1-IRES-GFP) and pCREB and the arrowheads neurons that express the transgene but not pCREB. **f** Quantification of percentage of IRES-GFP and Prox1-IRES-GFP neurons positive for pCREB ($n=4$ independent cultures). $^{**}p<0.01$, $^{***}p<0.001$. Scale bars: 75 μm

indirectly, and as a final outcome, it inhibits CREB phosphorylation, a downstream event in calcium signaling and a critical player in promoting neuronal maturation.

Consistent with a crucial role of Prox1 in neuronal maturation, human *PROX1* gene locus has been previously correlated with neurological and neurodevelopmental conditions. GWAS studies have shown that single-nucleotide polymorphisms (SNPs) from the Prox1 upstream gene regulatory sequences have been associated with Schizophrenia [41, 42] and Alzheimer's disease [43].

Conclusively, in this study, we have revealed a central role for Prox1 in neurite elongation, possibly through the negative regulation of calcium signaling pathway. Moreover, here, we uncovered a previously unknown effect of Prox1 on calcium signaling that may also apply in other tissues, where Prox1 plays pivotal roles in development, homeostasis, and function of organs such as heart, liver, pancreas, and lymphatics.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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Long non-coding RNA Lacuna regulates neuronal differentiation of neural stem cells during brain development

Elpinickie ninou[1], Artemis Michail[1], Panagiotis K. Politis[1*]

[1]Biomedical Research Foundation of the Academy of Athens (BRFAA), Greece

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Author contribution statement

E. Ninou and A. Michail performed the experiments, analyzed the data and prepared the manuscript. P. K. Politis conceived the project, designed and supervised the research and wrote the manuscript.

Keywords

Tbr2/Eomes, NONMMUT071331, non-coding genome, lncRNAs, KRAB/CRISPR/dCas9

Abstract

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Although long non-coding RNAs (lncRNAs) is one of the most abundant classes of RNAs encoded within the mammalian genome and are highly expressed in the adult brain, they remain poorly characterized and their roles in the brain development are not well understood. Here we identify the lncRNA Lacuna (also catalogued as NONMMUT071331.2 in NONCODE database) as a negative regulator of neuronal differentiation in the neural stem/progenitor cells (NSCs) during mouse brain development. In particular, we show that Lacuna is transcribed from a genomic locus near to the Tbr2/Eomes gene, a key player in the transition of intermediate progenitor cells towards the induction of neuronal differentiation. Lacuna RNA expression peaks at the developmental time window between E14.5 and E16.5, consistent with a role in neural differentiation. Overexpression experiments in ex vivo cultured NSCs from murine cortex suggest that Lacuna is sufficient to inhibit neuronal differentiation, induce the number of Nestin+ and Olig2+ cells, without affecting proliferation or apoptosis of NSCs. CRISPR/dCas9-KRAB mediated knockdown of Lacuna gene expression leads to the opposite phenotype by inducing neuronal differentiation and suppressing Nestin+ and Olig2+ cells, again without any effect on proliferation or apoptosis of NSCs. Interestingly, despite the negative action of Lacuna on neurogenesis, its knockdown inhibits Tbr2 transcription, implying a simultaneous, but opposite, role in facilitating the Tbr2 gene expression. Collectively, our observations indicate a critical function of Lacuna in the gene regulation networks that fine tune the rate of neuronal differentiation in the mammalian NSCs.

Contribution to the field

Although long non-coding RNAs (lncRNAs) is one of the most abundant classes of RNAs encoded within the mammalian genome and are highly expressed in the adult brain, they remain poorly characterized and their roles in neural stem cells and brain development are not well understood. In this article we: 1. characterize Lacuna, as a new long non-coding RNA expressed in the mouse neural stem cells, that is transcribed from the genomic locus of Tbr2/Eomes gene, a key player in the induction of neuronal differentiation. 2. show that Lacuna is differentially expressed during murine cortical development. 3. reveal a regulatory effect of Lacuna on Tbr2 gene expression in neural stem cells. 4. identify Lacuna as a negative regulator of neuronal differentiation in the neural stem cells during mouse brain development. Collectively, our observations indicate a critical function of Lacuna in the gene regulation networks that fine-tune the rate of neuronal differentiation in the mammalian neural stem cells. We feel that this study is of broad interest, especially to scientists working in the fields of Developmental Biology, RNA Biology, Cell Differentiation, Gene Regulation and Neuroscience.

Ethics statements

Studies involving animal subjects

Generated Statement: The animal study was reviewed and approved by Local ethics committee of Athens Prefecture Veterinarian Service, Athens, Greece (approval No: 5523/16-10-2018) and took place in the animal facilities of the Center for Experimental Surgery of the Biomedical Research Foundation of the Academy of Athens, Greece.

Studies involving human subjects

Generated Statement: No human studies are presented in this manuscript.

Inclusion of identifiable human data

Generated Statement: No potentially identifiable human images or data is presented in this study.

Data availability statement

Generated Statement: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Long non-coding RNA Lacuna regulates neuronal differentiation of neural stem cells during brain development

Elpinickie Ninou[1,2], Artemis Michail[1,3] and Panagiotis K. Politis[1]

[1]*Center for Basic Research, Biomedical Research Foundation of the Academy of Athens, 4 Soranou Efesiou Str., 115 27, Athens, Greece.*

[2]*Department of Biology, National and Kapodistrian University of Athens, Panepistimioupolis, 15784, Athens, Greece.*

[3]*Department of Biology, University of Patras, Panepistimioupolis Rio, 26500, Patras, Greece.*

Contact

Panagiotis K. Politis,

Center for Basic Research, Biomedical Research Foundation of the Academy of Athens, 4 Soranou Efesiou Str., 115 27, Athens, Greece

Tel: 0030-210-6597479; Fax: 0030-210-6597545

Email: ppolitis@bioacademy.gr

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Abstract

Although long non-coding RNAs (lncRNAs) is one of the most abundant classes of RNAs encoded within the mammalian genome and are highly expressed in the adult brain, they remain poorly characterized and their roles in the brain development are not well understood. Here we identify the lncRNA *Lacuna* (also catalogued as *NONMMUT071331.2* in NONCODE database) as a negative regulator of neuronal differentiation in the neural stem/progenitor cells (NSCs) during mouse brain development. In particular, we show that *Lacuna* is transcribed from a genomic locus near to the *Tbr2/Eomes* gene, a key player in the transition of intermediate progenitor cells towards the induction of neuronal differentiation. *Lacuna* RNA expression peaks at the developmental time window between E14.5 and E16.5, consistent with a role in neural differentiation. Overexpression experiments in *ex vivo* cultured NSCs from murine cortex suggest that *Lacuna* is sufficient to inhibit neuronal differentiation, induce the number of Nestin+ and Olig2+ cells, without affecting proliferation or apoptosis of NSCs. CRISPR/dCas9-KRAB mediated knockdown of *Lacuna* gene expression leads to the opposite phenotype by inducing neuronal differentiation and suppressing Nestin+ and Olig2+ cells, again without any effect on proliferation or apoptosis of NSCs. Interestingly, despite the negative action of *Lacuna* on neurogenesis, its knockdown inhibits *Tbr2* transcription, implying a simultaneous, but opposite, role in facilitating the *Tbr2* gene expression. Collectively, our observations indicate a critical function of *Lacuna* in the gene regulation networks that fine tune the rate of neuronal differentiation in the mammalian NSCs.

Introduction

Understanding the molecular mechanisms that control the mammalian brain development is one of the most challenging goals of biomedical sciences. For a long time, it was thought that an intricate network of transcription factors and chromatin modulators are solely responsible for activating or repressing specific genes or gene circuitries to control proliferation, differentiation and specification of neural stem/progenitor cells (NSCs) during the brain formation (Corbin et al., 2008; Martynoga, Drechsel, & Guillemot, 2012; Urban & Guillemot, 2014). However, the emergence of new genome sequencing technologies and the experimental data from large consortia such as ENCODE and FANTOM have radically changed our view of the organization, activity and regulation of the mammalian genome (Birney et al., 2007; Carninci et al., 2005; Consortium, 2012; Katayama et al., 2005). It has now become clear that most of the genome is transcribed and produces a large number of regulatory RNA molecules that were not previously known. Among them, long non-coding RNAs (lncRNAs) are transcripts larger than 200 nt that can be modified by 5'-capping, polyadenylation and splicing, similar to mRNAs, yet they are not translated into proteins (Djebali et al., 2012; Maeda et al., 2006). Their genomic location varies as they can be found into introns of protein coding genes, sense or anti-sense to other genes, intergenic regions (Kapranov et al., 2007; Seila et al., 2008), promoters (Hung et al., 2011), enhancers (Ørom et al., 2010), gene regulatory regions like UTRs (Mercer et al., 2011), even telomeres (Azzalin, Reichenbach, Khoriauli, Giulotto, & Lingner, 2007). Most importantly, lncRNAs appear to be involved in the regulatory networks that control stem cell pluripotency, carcinogenesis, growth, and function of many tissues and organs (Antoniou, Stergiopoulos, & Politis, 2014; Chi, Wang, Wang, Yu, & Yang, 2019; Giakountis et al., 2016; Guttman et al., 2011; Malissovas, Ninou, Michail, & Politis, 2019; Mercer, Dinger, & Mattick, 2009; Ng, Johnson, & Stanton, 2012; Ramos et al., 2015; Sheik Mohamed, Gaughwin, Lim, Robson, & Lipovich, 2010; Zarkou, Galaras, Giakountis, & Hatzis, 2018).

Likewise, many recent studies indicate that lncRNAs are involved in homeostasis and function of the mammalian brain as well as in the pathophysiology of brain related diseases, including neurodevelopmental disorders (Barry et al., 2014; Briggs, Wolvetang, Mattick, Rinn, & Barry, 2015; Elkouris et al., 2019; Faghihi et al., 2008; Hosseini, Bagheri-Hosseinabadi, De Toma, Jafarisani, & Sadeghi, 2019; Johnson, 2012; Tsagakis, Douka, Birds, & Aspden, 2020). Accordingly, it has been reported that thousands of lncRNAs are expressed in the embryonic and adult mammalian brain in a highly patterned and specific manner (Andersen & Lim, 2018; Fatica & Bozzoni, 2014; Ponjavic, Oliver,

Lunter, & Ponting, 2009), yet they remain poorly characterized and their roles in brain development have not been extensively studied. Towards this direction, it has been suggested that a significant proportion of lncRNAs may have the ability to regulate *in cis* the neighboring protein-coding genes via the reorganization of chromatin microenvironment (Bond et al., 2009; Sauvageau et al., 2013; Zhang et al., 2012). Along these lines, we have previously proposed that a subset of lncRNAs that are transcribed in close genomic proximity to genes encoding for transcription factors with critical roles in the brain development, may also be able to regulate these genes and therefore involved in neural development (Antoniou et al., 2014).

Here we identify *Lacuna* (also catalogued as *TCONS_00034309* or *NONMMUT071331.2* in NONCODE database) as a lncRNA gene localized near to the *Tbr2/Eomes* transcription factor gene, and that *Lacuna* is highly and differentially expressed during embryonic development of mouse cortex. Interestingly, *Lacuna* has not been previously studied in the context of nervous system or any other tissue, organ or cell type. By gain- and loss-of-function experiments in NSCs isolated from the murine embryonic cortex, we show that *Lacuna* suppresses neuronal differentiation, possibly via an *in trans* action. At the same time, *Lacuna* expression is required for the *Tbr2* gene expression, a function that is opposite to its negative role in neurogenesis, since *Tbr2* promotes neuronal differentiation (Arnold, Huang, et al., 2008; Englund et al., 2005; Hevner, 2019; Lv et al., 2019; Mihalas et al., 2016; Sessa et al., 2017). These two opposite functions may indicate that *Lacuna* is an interconnecting node in the gene regulatory networks that fine tune the rate of differentiation in NSCs during development.

Materials and Methods

Ethics statement

The study protocol took place in the animal facilities of the Center for Experimental Surgery of the Biomedical Research Foundation of the Academy of Athens. All animals were handled in strict accordance with good animal practice as defined by the relevant European and Greek animal welfare bodies.

Culture of NSCs, overexpression and knockdown studies

Neurosphere cultures from E14.5 mouse cortical tissue were prepared as previously described (Kaltezioti et al., 2014; Kaltezioti et al., 2010; Politis, Akrivou, Hurel, Papadodima, & Matsas, 2008) (Stergiopoulos & Politis, 2016). Proliferation or differentiation assays were performed after dissociation of NSCs to single cells, plating onto poly-L-lysine (Sigma) coated coverslips in 24-well plates at a density of $1x10^5$ and further ex vivo culture for 2 or 3 days with or without Growth Factors, respectively, in a 37° C humidified incubator with 5% CO_2. The cells were maintained in suspension in full medium with growth factors as follows: 1:1 mixture of Dulbecco's modified Eagle's medium (1 g/liter d-glucose, l-glutamine, pyruvate; Sigma), F-12 nutrient mixture (Sigma) plus 20 ng/ml human epidermal growth factor (EGF; R&D Systems) and 20 ng/ml human basic fibroblast growth factor (R&D Systems), 20 μg/ml insulin (Sigma), 1x B27 supplement (Gibco), 0.25 mM l-glutamine, and 1% penicillin/streptomycin to promote the production of floating neurospheres. The neurospheres were then passaged 3–4 times before the assays. Differentiated neurosphere cultures were maintained in minus growth factors conditions, the same as the full medium plus growth factors without human EGF and basic FGF, in order to promote differentiation.

For overexpression studies, the *Lacuna* lncRNA sequence was first cloned into pcDNA3.1 (GenScript) and then it was sub-cloned into pCAGGs vector. Together with pCAGGs-*Lacuna* a pCAGGs-GFP plasmid was used (in a ratio of 3:1) in order to visually mark the transfected cells. Empty pCAGGs together with pCAGGs-GFP (in a ratio of 3:1) were also used as a control for the overexpression experiments.

For knockdown studies, a CRISPR-dCas9-KRAB effector system was used, kindly provided by Dr. Pantelis Hatzis (BSRC, Al Fleming, Athens Greece). This system consists of two plasmids (1:1): a pHR-KRAB-dCas9-mCherry and a pU6-sgRNA-EF1Alpha-puro-T2A-BFP (without gRNA for control and with gRNAs against *Lacuna* sequence for *Lacuna* knockdown). gRNAs were designed using the GenCRISPR gRNA Design Tool (https://www.genscript.com/gencrispr-grna-design-tool.html?src=google&gclid=CjwKCAjwn6GGBhADEiwAruUcKty8qKnSWhOxCpac_VrRqHDGm4a7RgBDp01gPjihJLS0Ydvtzw482BoC7WMQAvD_BwE) to target the first exon of *Lacuna* sequence.

NSCs were transfected using an AMAXA electroporator (Lonza) with 6μg of total plasmid DNA per electroporation, according to manufacturer's instructions, as also previously described (Kaltezioti et al., 2014; Kaltezioti et al., 2010). After electroporation, NSCs were incubated overnight in full

medium with 1% FBS in order for them to surpass electroporation shock and then they were incubated according to the experiment.

RNA extraction and real-time RT-qPCR analysis

Total RNA was isolated by cells and tissues with TRI reagent solution (AM9738, Ambion/RNA, Life Technologies) according to manufacturer's instructions, followed by treatment with RQ1 DNase (Promega, Madison, WI, USA). RNA concentration and purity was measured by Nanodrop 2000c (Thermo), and 1.5 μg of RNA was used for cDNA synthesis using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, USA) together with random hexamer primers. Quantitative Real time RT-PCR analysis was performed in a LightCycler 96 Instrument (Roche). Measured values were normalized using beta actin or Gapdh and RPL13A mRNA levels as internal references.

Primers that were used for real-time RT-qPCR are presented in the following table:

Genes	**Sequence**	
beta actin	Forward	CCCAGGCATTGCTGACAG
	Reverse	TGGAAGGTGGACAGTGAGGC
Gapdh	Forward	TGCCACTCAGAAGACTGTGG
	Reverse	TTCAGCTCTGGGATGACCTT
RPL13A	Forward	ATGACAAGAAAAAGCGGATG
	Reverse	CTTTTCTGCCTGTTTCCGTA
Tbr2	Forward	TTCCGGGACAACTACGATTCA
	Reverse	ACGCCGTACCGACCTCC
Lacuna	Forward	CGGGTCCTCTCAAGTCAGTC
	Reverse	GTTGCTTCCACATGCTTCCT
Golga4	Forward	GTTGAAGCACACGTCCACAC
	Reverse	AGTTCGGCTTCCACCTCTTG
Gm33450	Forward	GGAGGACGGGAAAGACTGTC
	Reverse	TTGTTGTAGGGCTGGCTCTG
U6long	Forward	GTGCTCGCTTCGGCAGCACA
	Reverse	GGAACGCTTCACGAATTTGCGTGTCAT
18s	Forward	TTGACGGAAGGGCACCAC
	Reverse	ACCACCACCCACGGAATC

7SK	Forward	TTCCCCGAATAGAGGAGGAC
	Reverse	GCCTCATTTGGATGTGTCTG

RT-PCR Mapping of Lacuna transcript

As the *Lacuna* lncRNA is not yet annotated, we specified the boundaries of its three exons using appropriate primers and PCR (KAPA Taq PCR Kit). The templates were cDNAs derived from RNA of embryonic mouse cortices in embryonic days E12, E14, E16, E18 and newborns P0. Then, we performed gel electrophoresis of PCR products using the appropriate DNA ladder (Quick Load Purple 100 bp DNA Ladder, #NO551G, New England Biolabs).

Primers that were used for PCR are presented in the following list:

Forward Primers:

Primer-1: CTGGCACTGAGTACTCTGGGGACCCAAC

Primer-2: ACTCTGGGGACCCAACTTTT

Primer-3: CGGGTCCTCTCAAGTCAGTC

Primer-4: AAATCTCCACCGGGTGAAAG

Reverse Primers

Primer-5: GTGGGCTTCATTTCTTCAGC

Primer-6: GTTGCTTCCACATGCTTCCT

Primer-7: GTCTATTTCAAGTCTTGTATATTTTTGCACCG

Subcellular fractionation

Neurospheres were cultured in full medium plus growth factors and were harvested in passage 2. Subcellular fractionation was performed with PARIS Kit Protein and RNA isolation system (Ambion, AM1921). Nuclear and cytoplasmic samples were obtained and then, we performed RNA isolation, cDNA synthesis and real time RT-qPCR analysis. Efficient fractionation of the subcellular compartments and normalization of the measured values were evaluated by using Gapdh, U6long, 18s and 7sk primer pairs.

Immunofluorescence

For the cell immunostaining experiments, primary cells were cultured onto poly-L-lysine (Sigma) coated coverslips in 24-well plates. In particular, after electroporation, NSCs were cultured for 24h in full medium (+GF) to recover from the electroporation reaction. Then, these cells were cultured either in the presence of growth factors for 48h (to measure proliferation and associated markers), or in the absence of growth factors for 72h (to measure differentiation and associated markers). At the end of the experiment, cells were fixed on the coverslips with 4% PFA. The coverslips were blocked with 5% FBS in 1x PBS, containing 0.3% triton X-100 for 2 hours at room temperature (RT). Next, they were incubated with primary antibodies at 4° C overnight, followed by secondary antibodies for 2 hours at RT. Finally, they were incubated with DAPI, diluted in 1X PBS for 10min at RT, followed by mounting with MOWIOL. The primary antibodies in the immunofluorescence were anti-BrdU (Abcam, 6326) (1:400 dilution), rabbit anti-cleaved caspase 3 (Cell Signaling, 9661) (1:800 dilution), mouse Tuj1/anti-beta III tubulin (Covance, MMS-435P-250) (1:1000 dilution), anti-GFAP (Abcam, 4674) (1:1500 dilution), rabbit anti-Tbr2 (Abcam, Ab23345) (1:1000 dilution), goat anti-Olig2 (1:400 dilution), mouse anti-NeuN (Millipore, MAB337) (1:200 dilution), chicken anti-GFP (Abcam, Ab13790), chicken anti-mCherry (Novus, NBP2-25158) (1:1000 dilution). The secondary antibodies were donkey anti-Rabbit 488 (AlexaFluor), donkey anti-Mouse 568 (AlexaFluor), donkey anti-Rabbit 647 (AlexaFluor), donkey anti-Chicken 488 (AlexaFluor), donkey anti-Rat 488 (AlexaFluor).

In Situ Hybridization on Cryosections

Mouse embryonic brains of various developmental stages were incubated in 4% PFA for 4 hours and left overnight in 30% sucrose in PBS for cryoprotection. Then, the tissue was embedded in OCT, sectioned transversely at 12μm and collected on super-frost slides. Non-radioactive in situ hybridization on cryosections were carried out as previously described (Kaltezioti et al., 2014; Kaltezioti et al., 2010). The RNA probes complementary to *Lacuna* were prepared and labeled with digoxigenin.

Statistical analysis

All experimental designs are explained in each part of the section "materials and methods", respectively. The normal distribution of values was verified with the Shapiro–Wilk normality test using IBM SPSS Statistics for Windows, Version 20.0. To ensure the reproducibility of results, all experiments were performed independently three to four times as indicated in each figure legend. For statistical analysis all measurements and experimental values from independent experiments were estimated with two-tailed Student's t-test or two-way ANOVA. All the results are shown as mean ± SD. The exact P values are described in each figure legend. P values < 0.05 are considered statistically significant. All analyses were done using Microsoft Excel 2013 and GraphPad Prism 8.

Results

Lacuna lncRNA is expressed in the developing murine cortex

We and others had previously reported that a number of lncRNA genes are found in close genomic proximity (less than a 2kb distance) to genes encoding for transcription factors with critical regulatory roles in neural development (Antoniou et al., 2014; Ponjavic et al., 2009). We hypothesized that a subset of these lncRNAs may be also involved in neural development by directly affecting the expression of neighboring protein coding genes. Thus, we decided to focus on such a pair of transcription factor/lncRNA genes, and more specifically on the *Lacuna* lncRNA, which is 1661 nt long (sequence information in Supplementary Figure 1A) and transcribed from a genomic locus, only 1.5 kb far from the *Tbr2/Eomes* gene (Figure 1A). *Lacuna* has not been previously studied or reported in the literature, the only reference for this transcript is its presence in the RNA-seq databases from large scale consortia, where it is catalogued as *TCONS_00034309* or *NONMMUT071331.2* (NONCODE database). We have renamed this transcript and corresponding gene as *Lacuna*. RNA-seq data from NONCODE suggest expression in various adult mouse tissues, including heart, liver, lung, spleen, thymus and hippocampus (Supplementary Figure 1B). On the other hand, the protein coding gene of the pair, the *Tbr2* gene, encodes a transcription factor with a well-established role in promoting neuronal differentiation in cortical development (Arnold, Huang, et al., 2008; Hevner, 2019; Mihalas et al., 2016; Sessa et al., 2017; Vasistha et al., 2015). An intriguing question arising from these observations is whether *Lacuna* is playing any role in neural development either via an *in cis* effect on *Tbr2* gene expression or an independent function.

To tackle this question, we initially investigated the expression pattern of *Lacuna* in the murine cortex during development. In particular, by real time RT-qPCR assays, we showed that *Lacuna* is differentially expressed during cortical development, with its peak of expression to be observed in the time window between E14 and E16, and then declines dramatically in E18 and P0 (Figure 1B). Its expression pattern is similar to the expression pattern of the neighboring *Tbr2* gene (Figure 1B-C), although a shift towards later developmental stages is also evident in the case of *Lacuna*. As Lacuna is not yet annotated, we wanted to verify its RNA sequence and its exon-to-exon junctions, as reposited in the NONCODE database (Supplementary Figure 1A). To do that, we designed multiple sets of primers specific for the exon-exon boundaries as well as for amplicons including a combination of exons in order to verify the reposited sequence (Supplementary Figure 2A). We used these primers in RT-PCR reactions using as a starting material total cortical RNA from different developmental stages. All RT-PCR reactions produced products compatible with the sequence of *Lacuna* as reposited in NONCODE database (Supplementary Figure 2A). In addition, we showed that *Lacuna* is not subjected to alternative splicing in mouse developing cortex, as there are the expected exon-to-exon junctions and none of the exons is skipped during splicing (Supplementary Figure 2B). Next, *in situ* hybridization experiments confirmed the expression of *Lacuna* in the E16 and E14 murine cortex (Figure 1D), indicating a pattern of expression spanning the VZ/SVZ as well as outer cortical layers.

Furthermore, to define the subcellular localization of *Lacuna*, we performed subcellular fractionation of NSCs in conjunction with real time RT-qPCR. These NSCs were isolated from murine cortex at E14 and cultured *ex vivo*. Accordingly, we were able to show that *Lacuna* is localized both in the cytosol and the nucleus (Figure 1E). Control reactions for *U6* RNA and *Gapdh* mRNA indicate that our nuclear and cytosolic fractions, respectively, were efficiently separated, further confirming our observation for *Lacuna*. In agreement, analysis of higher magnification images from the *in situ* hybridization experiments on E16 mouse embryonic cortex nicely corroborated these data, by showing distribution of *Lacuna* in both cellular compartments (Figure 1F). Therefore, *Lacuna* RNA is found both at cytoplasm and nucleus, indicating that this molecule may exert nuclear and/or cytoplasmic roles.

Lacuna overexpression inhibits neuronal differentiation of NSCs

To gain insights into the functional role of *Lacuna* in neurodevelopment, we first studied the effect of its overexpression on *ex vivo* cultured NSCs, isolated from E14 murine cortex. Specifically, we

constructed plasmids that were able to efficiently overexpress *Lacuna* and GFP under the CAGG promoter, which works well with mammalian cells as we have previously reported (Kaltezioti et al., 2021; Kaltezioti et al., 2010; Stergiopoulos & Politis, 2016). A mixture of two plasmids, pCAGGs-*Lacuna* and pCAGGs-*GFP* (experimental condition) or pCAGGS empty and pCAGGS-*GFP* (control condition), was used to transfect NSCs with Amaxa electroporation technique (Figure 2A). In addition, by using Amaxa electroporation system, we have previously established and reported methodologies to perform gain- and loss-of-function experiments in embryonic NSCs as well as to extensively investigate the contribution of genes and molecular players in proliferation, differentiation, specification, and maturation of neural cells (Kaltezioti et al., 2014; Kaltezioti et al., 2010; Stergiopoulos & Politis, 2016), as also schematically described in Figure 2A.

Accordingly, we found that *Lacuna* overexpression is not affecting proliferation or apoptosis of *ex vivo* cultured NSCs (Figure 2B-E), yet it is sufficient to significantly induce the numbers of Nestin+ cells, a marker of neural cell stemness (Figure 2F-G). Remarkably, *Lacuna* overexpression caused a significant reduction in the ability of NSCs to produce βIII-tubulin+ neurons (Figure 3A-B) and NeuN+ neurons (Figure 3C-D) under differentiation conditions [without growth factors (GF)] as compared to the control condition. Interestingly, astrogliogenic differentiation (GFAP marker) remains unaffected under *Lacuna* overexpression (Figure 3E-F). However, we found a *Lacuna*-mediated increase in the population of Olig2+ cells (Figure 3G-H). We assume that this extra population corresponds to Olig2-expressing neural progenitor cells that are not able to differentiate into neurons. Therefore, these observations suggest that *Lacuna* is sufficient to exert a significant effect on the ability of NSCs to differentiate into neurons without affecting astrogliogenic or proliferative capacities of these cells.

Lacuna knockdown promotes differentiation of NSCs

To further investigate the involvement of *Lacuna* in NSCs fate decision, we assessed whether it is necessary for NSCs differentiation by performing knockdown experiments using a CRISPR-dCas9-KRAB effector system (Alerasool, Segal, Lee, & Taipale, 2020; Parsi, Hennessy, Kearns, & Maehr, 2017). This system is highly effective and specific in knocking down lncRNAs expression, but at the same time it leaves DNA intact, meaning that there are no changes at the level of DNA sequence (Figure 4A), as is the case with the traditional CRISPR-Cas9 methodology. This feature is extremely helpful in the case of lncRNAs research, since it has been previously shown that deletion of lncRNAs genomic loci may lead

to significant effects on cellular functions due to the DNA changes (e.g. deletion of regulatory DNA elements) and not due to the downregulation of lncRNA expression (Kopp & Mendell, 2018; Paralkar et al., 2016). To achieve the CRISPR-dCas9-KRAB-mediated knockdown of *Lacuna* RNA expression, we utilized 3 different guide RNAs (sgRNAs). All of them have been designed in such a way (GenCRISPR gRNA Design Tool) to target the first exon of *Lacuna* gene. Thus, we showed that all three of them are able to downregulate the expression of *Lacuna* RNA (Figure 4B), so we continued our studies with the gRNA that had the strongest effect.

In good agreement with the overexpression studies, proliferation and apoptosis are not affected by *Lacuna* knockdown in NSCs (Figure 4C-F), while the number of Nestin+ cells is significantly decreased (Figure 4G-H). Conversely to the overexpression studies, *Lacuna* knockdown in primary NSCs resulted in a significant increase of β-III tubulin+ neurons (Figure 5A-B) and NeuN+ neurons (Figure 5C-D), but also of GFAP+ astrocytes (Figure 5E-F), as shown by immunofluorescent experiments in the absence of GFs. The Olig2+ population was found decreased (Figure 5G-H), hence exhibiting an opposite effect than that of *Lacuna* overexpression condition. Taken together, these observations indicate that *Lacuna* RNA is critically involved in the regulation of neuronal differentiation in NSCs.

Lacuna is necessary for _Tbr2/Eomes_ expression in NSCs

Next, we wanted to investigate whether the effect of *Lacuna* on NSCs is mediated through a possible action on the *Tbr2/Eomes* gene expression. Since it has been previously reported that *Tbr2* facilitates neuronal differentiation, we would expect a negative action of *Lacuna* on *Tbr2* expression. Towards this direction, we examined whether knockdown of *Lacuna* affects the mRNA expression of *Tbr2/Eomes* and other genes in its genomic neighborhood. First, we focused on the other genes of the locus to confirm the specificity of our approach. Accordingly, we searched for possible effects on neighboring to *Lacuna* genes and specifically, on *Golga4* gene and a recently annotated non-coding RNA gene, *Gm33460* (Figure 6A). *Golga4* is approximately 16500 bp away from the 5' of *Lacuna* and it encodes one of the golgins, a family of proteins localized in the Golgi apparatus. *Gm33460* is downstream to *Lacuna* with a small common sequence shared between these two transcripts (end of 2nd exon and beginning of 3rd exon), but it continues after the RNA sequence of *Lacuna* (Figure 6A). Notably, both *Golga4* and *Gm33460* are not affected by KRAB-dCas9 that is targeted to *Lacuna* sequence, as shown by real time RT-qPCR assays (Figure 6B-E). These observations suggest that our

knockdown approach is specific and sufficient to downregulate *Lacuna* expression, without affecting the other two genes, which are found close to *Lacuna* transcription start site (TSS).

Surprisingly, upon knockdown of *Lacuna* in NSCs and under minus growth factor conditions, *Tbr2/Eomes* gene expression is downregulated (Figure 6B-C). Consistently, knockdown of *Lacuna* induces a statistically significant downregulation of the *Tbr2* expression at the protein level as well, as shown with immunofluorescence experiments (Figure 6F-G). On the other hand, *Lacuna* overexpression does not affect the numbers of Tbr2+ cells, nor *Tbr2* gene expression at the mRNA level (Figure 6H-K). This difference between knockdown and overexpression experiments probably indicates that *Lacuna* is able to regulate *Tbr2/Eomes* gene only *in cis*. Thus, only when we are knocking down the cis-expressed *Lacuna* gene, we are observing an effect on *Tbr2/Eomes* gene expression. On the other way round, when we are providing *Lacuna* transcript *in trans*, by exogenously overexpressing it, we are not able to detect any effect on *Tbr2/Eomes* gene expression. Consequently, we favor a conclusion that *Lacuna* is positively regulating the expression of *Tbr2/Eomes in cis*.

However, this positive action cannot explain the effect of *Lacuna* on neuronal differentiation, since *Tbr2* promotes neuronal differentiation (Arnold, Hofmann, Bikoff, & Robertson, 2008; Arnold, Huang, et al., 2008; Englund et al., 2005; Sansom et al., 2009). Therefore, *Lacuna*-mediated regulation of *Tbr2/Eomes* gene expression cannot explain its role in inhibiting neuronal differentiation. In agreement, *Lacuna* overexpression can inhibit neuronal differentiation without affecting *Tbr2/Eomes* expression. Thus, we propose a hypothetical model where *Lacuna* exerts a *Tbr2*-independent effect on differentiation via a mechanistic action in the nucleus and/or in the cytoplasm.

Discussion

The complexity of the mammalian brain is mainly due to the huge numbers of neurons and glial cells that interact to form its underlying structure. All these cells are derived from a pool of neural stem cells that proliferate with enormous rates and then differentiate to generate first neurons and then glial cells. The differentiation of neural stem cells towards the neuronal or glial cell identity is a major developmental process controlled by the interplay between extracellular signaling cues and intrinsic gene regulation networks (Gallo & Deneen, 2014; Guérout, Li, & Barnabé-Heider, 2014; Lalioti et al.,

2019; Martynoga et al., 2012; Okawa, Nicklas, Zickenrott, Schwamborn, & Del Sol, 2016; Paridaen & Huttner, 2014; Segklia et al., 2012; Urban & Guillemot, 2014). Elucidation of the molecular mechanisms that control these networks could provide valuable information on how the mammalian brain is formed as well as useful insights into the involvement of new molecular players in nervous system diseases, disorders and cancers. It has recently become evident that a large part of the non-coding genome is transcribed producing RNA molecules that are not translated into proteins, but they exhibit tissue and cell-type specific patterns of expression (Djebali et al., 2012; Fatica & Bozzoni, 2014; Maeda et al., 2006). Among them, lncRNAs represent a large part of the mammalian genes and according to some estimations larger than the part of protein-coding genes (Carninci et al., 2005; Hosseini et al., 2019). Intriguingly, the lncRNAs that are expressed in the mammalian brain are preferentially harbored by genomic loci in the vicinity of brain-specific, transcriptionally active during development, protein-coding genes (Antoniou et al., 2014; Ponjavic et al., 2009). Here we wanted to investigate the relationship between lncRNAs and protein-coding genes, therefore we decided to focus on the *Tbr2* genomic locus. From this locus, the *Lacuna* lncRNA is transcribed in a close genomic proximity to the gene encoding for the transcription factor *Tbr2*. The rationale for choosing *Tbr2-Lacuna* genomic locus is the fact that *Tbr2* is a key player in neuronal differentiation during cortical neurogenesis (Arnold, Huang, et al., 2008; Englund et al., 2005; Hevner, 2019; Sessa et al., 2017; Vasistha et al., 2015). On the other hand, almost nothing was known about *Lacuna* in the nervous system or other tissues or cell types. We showed here that *Lacuna* expression is significantly induced in the murine embryonic cortex at E14 and remains high until E16, to be then reduced at E18 and P0. *Lacuna* expression is similar to that of *Tbr2* (Bulfone et al., 1999; Englund et al., 2005; Kimura, Nakashima, Ueno, Kiyama, & Taga, 1999; Vasistha et al., 2015), suggesting a common regulation of these two genes or a synergistic interaction between them. Indeed, with our knockdown strategy in primary NSC cultures, we revealed that *Lacuna* is necessary for *Tbr2* gene expression. However, exogenous overexpression of *Lacuna* is not sufficient to upregulate or in any way affect *Tbr2* expression in NSCs. These observations indicate that *Lacuna* transcript can regulate *Tbr2* expression only *in cis*. The *in cis* action of *Lacuna* is also supported by the presence of *Lacuna* transcripts in the nucleus of NSCs, indicating a function related to the regulation of gene expression. *Lacuna* is also equally distributed between nucleus and cytoplasm suggesting that it has additional roles in the cytoplasmic compartment.

Tbr2 is transiently expressed in the cortical progenitor cells during embryonic development to promote neuronal differentiation (Arnold, Huang, et al., 2008; Englund et al., 2005). Based on these data and the positive role of *Lacuna* in *Tbr2* gene expression, it could be assumed that *Lacuna* may also promote neuronal differentiation of NSCs. Surprising enough, we show that *Lacuna* exerts exactly the opposite action by inhibiting neurogenesis (βIII-tubulin and NeuN markers) and promoting a neural stem cell-like identity (Nestin and Olig2 markers). This unexpected finding suggests that *Lacuna* affects neuronal differentiation via a *Tbr2*-independent (*in trans*) mechanistic function on other gene(s) or pathways. Consistent with this scenario, overexpression of *Lacuna* inhibits neuronal differentiation without influencing *Tbr2* expression. Yet, how *Lacuna* suppresses neuronal differentiation is still an open question. To this end, it is tempting to speculate that this effect of *Lacuna* is mediated by promoting the expression of *Olig2*. In agreement with this hypothesis, neurogenesis defects in *Lacuna* overexpressing NSCs are accompanied by a striking increase in the Olig2+ cells. The exact opposite effect on the numbers of Olig2+ cells were observed in NSCs that were lacking *Lacuna*. In accordance, it has been reported that Olig2 overexpression in neural stem cells elicits neurogenesis defects (Liu et al., 2015) and that Olig2 has also anti-neuronal functions in different developmental stages and depending on its phosphorylation state (Sun et al., 2011). Moreover, it is known that Olig2 antagonizes Ngn2 and inhibits the premature expression of post-mitotic motor neuron genes holding progenitor cells in reserve for later differentiation (Lee, Lee, Ruiz, & Pfaff, 2005). Altogether, these observations may indicate that *Lacuna* is involved in the regulation of neurogenesis, probably through an Olig2-mediated pathway.

Moreover, the fact that two genes from the same genomic locus are co-expressed with a similar pattern, yet they exert opposite roles, may indicate that positive and negative effectors of a cellular phenomenon are co-regulated to fine-tune the final outcome. Therefore, it could be that a pro-neurogenic factor, such as *Pax6*, may induce the transcriptional activation of the chromatin domain that includes both genes to primarily promote the expression of *Tbr2*, which in turn enhances neurogenesis. Simultaneously, a second transcript is produced from the same activation event, the *Lacuna* lncRNA, which partially counteracts the pro-neurogenic function of *Tbr2*, to fine-tune the number of neurons that are produced from a given pool of NSCs or alternatively to delay the depletion of NSC pool and maintain their differentiation potential for longer time periods. This hypothetical

scenario may point to a new emerging paradigm in genome science, where lncRNAs are co-regulated with protein coding genes with opposite functions to fine-tune the cellular action of the latter.

Author Contributions

E. Ninou and A. Michail performed the experiments, analyzed the data and prepared the manuscript. P. K. Politis conceived the project, designed and supervised the research and wrote the manuscript.

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Figure Legends

Figure 1. *Lacuna* is expressed in the mouse brain during embryonic development. (A) Schematic representation of *Lacuna* and *Tbr2/Eomes* locus. **(B)** RNA levels of *Lacuna* during mouse embryonic brain development. **(C)** mRNA levels of *Tbr2/Eomes* during mouse embryonic brain development. **(D)** *In situ* hybridization of *Lacuna* in cryosections from E16 and E14 mouse embryonic brain with the corresponding controls, as indicated. **(E)** Subcellular fractionation of NSCs and RNA levels of *Lacuna* in each subcellular compartment. mRNA levels of *U6* and *Gapdh* were used to verify the fractionation of cells (**p<0.01, n=3). **(F)** Higher magnification micrographs of the *In situ* hybridization experiment with *Lacuna* specific riboprobe in E16 mouse embryonic brain (left panel). Image in the right panel depicts larger magnification of the area included in the square shape of the image in the left panel. Asterisks indicate representative cells where the *Lacuna* is localized both in cytosol and nucleus.

Figure 2. *Lacuna* overexpression affects stemness but not proliferation nor apoptosis of mouse Neural Stem Cells. (A) Schematic representation of our experimental strategy. NSCs are derived from E14 mouse cortices and they are cultured appropriately to form neurospheres over 3 – 4 passages. Neurospheres are transfected with plasmids of choice and then, they are dissociated and plated. In the presence of growth factors, NSCs proliferate, whereas without growth factors, they differentiate to generate neurons and astrocytes. **(B)** *Lacuna-GFP* and *Control-GFP* transfected Neural Stem cells were treated with BrdU for 2h and then fixed and stained with anti-BrdU antibody (red), anti-GFP antibody (green) and 4, 6-diamidino-2-phenylindole (DAPI). Arrows indicate BrdU/GFP double positive cells. Scale bar: 250 μm. **(C)** Quantification of BrdU incorporation in transgene positive mouse Neural Stem cells (GFP: 19.55 ± 3.104%, Lacuna: 13.06 ± 1.098%, p>0.05). **(D)** *Lacuna-GFP* and *Control-GFP* transfected mouse Neural Stem cells were immunostained for cleaved caspase 3 (red), and GFP (green) and labeled with DAPI. Scale bar: 250 μm. **(E)** Quantification of cleaved caspase 3 positive cells in transgene positive mouse Neural Stem cells (GFP: 5.629 ± 1.409%, Lacuna: 10.05 ± 2.144%, p>0.05). **(F)** *Lacuna-GFP* and *Control-GFP* transfected mouse Neural Stem cells were immunostained for Nestin (red), GFP (green) and labeled with DAPI. Arrows indicate Nestin/GFP double positive cells. Scale bar: 100 μm. **(G)**

Quantification of Nestin positive cells in transgene positive mouse Neural Stem cells (GFP: 28.59 ± 2.691%, Lacuna: 43.55 ± 4.019%, p<0.01). For all cases, * p<0.05, ** p<0.01, *** p<0.001, n=3.

Figure 3. *Lacuna* overexpression inhibits neuronal differentiation of mouse Neural Stem Cells. (A) *Lacuna-GFP* and *Control-GFP* transfected mouse Neural Stem cells were immunostained for β-III tubulin (red) and GFP (green) and labeled with DAPI. Arrows indicate β-III tubulin/GFP double positive cells. Scale bar: 100 µm. **(B)** Quantification of β-III tubulin positive cells in transgene positive mouse Neural Stem cells (GFP: 46.82 ± 4.154%, Lacuna: 34.73 ± 3.399%, p<0.05). **(C)** *Lacuna-GFP* and *Control-GFP* transfected mouse Neural Stem cells were immunostained for NeuN (red), GFP (green) and labeled with DAPI. Arrows indicate NeuN/GFP double positive cells. Scale bar: 100 µm. **(D)** Quantification of NeuN positive cells in transgene positive mouse Neural Stem cells (GFP: 39.35 ± 4.721%, Lacuna: 13.37 ± 5.353, p<0.01). **(E)** *Lacuna-GFP* and *Control-GFP* transfected mouse Neural Stem cells were immunostained for GFAP (red), GFP (green) and labeled with DAPI. Scale bar: 100 µm. **(F)** Quantification of GFAP positive cells in transgene positive mouse Neural Stem cells (GFP: 55.50 ± 7.372%, Lacuna: 38.90 ± 6.521%, p>0.05). **(G)** *Lacuna-GFP* and *Control-GFP* transfected mouse Neural Stem cells were immunostained for Olig2 (red), GFP (green) and labeled with DAPI. Arrows indicate Olig2/GFP double positive cells. Scale bar: 100 µm. **(H)** Quantification of Olig2 positive cells in transgene positive mouse Neural Stem cells (GFP: 6.961 ± 1.905%, Lacuna: 21.00 ± 2.387%, p<0.05. For all cases, * p<0.05, ** p<0.01, *** p<0.001, n=3.

Figure 4. *Lacuna* knockdown reduces stemness but does not affect proliferation nor apoptosis of mouse Neural Stem Cells. (A) Scheme of dCas9-KRAB effector system and Lacuna knockdown strategy. The first plasmid expresses the guide RNAs that target *Lacuna*, the second plasmid expresses *dCas9-KRAB* and mcherry. When transfected together in Neural Stem cells, guide RNA recruits dCas9-KRAB fusion protein to Lacuna and inhibits its expression. In control cultures, NSCs were transfected with both plasmids, but first plasmid lacked a guide RNA sequence. **(B)** Three different guide RNA sequences were used to target *Lacuna* gene. All constructs were efficient in knocking down *Lacuna* expression. We selected sgRNA1 to proceed further. **(C)** Mouse Neural Stem cells were transfected with dCas9-KRAB-

mcherry and sgRNA1 targeting *Lacuna* (Lacuna KD-mcherry) or no guide RNA (CTRL-mcherry). They were treated with BrdU for 2h and then fixed and stained with anti-BrdU antibody (green), anti-mcherry (red) and 4, 6-diamidino-2-phenylindole (DAPI). Scale bar: 100 µm. **(D)** Quantification of BrdU incorporation in dCas9-KRAB-mcherry positive mouse Neural Stem cells (Control: 29.67 ± 2.63%, Lacuna KD: 30.18 ± 1.115%, $p>0.05$). **(E)** Mouse Neural Stem cells were transfected with dCas9-KRAB-mcherry and sgRNA1 targeting *Lacuna* (Lacuna KD-mcherry) or no guide RNA (CTRL-mcherry). They were immunostained for cleaved caspase 3 (green), mcherry (red) and labeled with DAPI. Scale bar: 100 µm. **(F)** Quantification of cleaved caspase 3 positive cells in dCas9-KRAB-mcherry positive mouse Neural Stem cells (Control: 3.261 ± 1.013%, Lacuna KD: 1.448 ± 0.4918%, $p>0.05$) **(G)** Mouse Neural Stem cells were transfected with dCas9-KRAB-mcherry and sgRNA1 targeting *Lacuna* (Lacuna KD-mcherry) or no guide RNA (CTRL-mcherry). They were immunostained for Nestin (green), mcherry (red) and labeled with DAPI. Scale bar: 100 µm. **(H)** Quantification of Nestin positive cells in dCas9-KRAB-mcherry positive mouse Neural Stem cells (Control: 43.99 ± 5.664%, Lacuna KD: 29.67 ± 2.176%, $p<0.05$). For all cases, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, $n=3$.

Figure 5. *Lacuna* knockdown promotes differentiation of mouse Neural Stem Cells. (A) Mouse Neural Stem cells were transfected with dCas9-KRAB-mcherry and sgRNA1 targeting *Lacuna* (Lacuna KD-mcherry) or no guide RNA (CTRL-mcherry). They were immunostained for β-III tubulin (green), mcherry (red) and labeled with DAPI. Scale bar: 100 µm. **(B)** Quantification of β-III tubulin positive cells in dCas9-KRAB-mcherry positive mouse Neural Stem cells (Control: 13.54 ± 1.481%, Lacuna KD: 21.50 ± 2.617%, $p<0.05$). **(C)** Mouse Neural Stem cells were transfected with dCas9-KRAB-mcherry and sgRNA1 targeting *Lacuna* (Lacuna KD-mcherry) or no guide RNA (CTRL-mcherry). They were immunostained for NeuN (green), mcherry (red) and labeled with DAPI. Scale bar: 100 µm. **(D)** Quantification of NeuN positive cells in dCas9-KRAB-mcherry positive mouse Neural Stem cells (Control: 31.86 ± 3.062%, Lacuna KD: 42.86 ± 2.723, $p<0.05$) **(E)** Mouse Neural Stem cells were transfected with dCas9-KRAB-mcherry and sgRNA1 targeting *Lacuna* (Lacuna KD-mcherry) or no guide RNA (CTRL-mcherry). They were immunostained for GFAP (green), mcherry (red) and labeled with DAPI. Arrows indicate GFAP/mcherry double positive cells. Scale bar: 100 µm. **(F)** Quantification of GFAP positive cells in dCas9-KRAB-mcherry positive mouse Neural Stem cells (Control: 17.43 ± 1.124%, Lacuna KD: 24.94 ± 1.165%, $p<0.001$) **(G)**

Mouse Neural Stem cells were transfected with dCas9-KRAB-mcherry and sgRNA1 targeting *Lacuna* (Lacuna KD-mcherry) or no guide RNA (CTRL-mcherry). They were immunostained for Olig2 (green), mcherry (red) and labeled with DAPI. Scale bar: 100 µm. **(H)** Quantification of Olig2 positive cells in dCas9-KRAB-mcherry positive mouse Neural Stem cells (Control: 79.59 ± 1.394%, Lacuna KD: 49.53 ± 3.023%, p>0.001). For all cases, * p<0.05, ** p<0.01, *** p<0.001, n=3.

Figure 6. *Lacuna* is necessary for *Tbr2/ Eomes* expression in mouse Neural Stem Cells. (A) Scheme of *Lacuna* and *Tbr2/Eomes* locus on mouse chromosome 9. Despite their vicinity, *Gm33460* and *Golga4* are not affected by guide RNAs targeting *Lacuna*. **(B)** RNA levels of lncRNA *Lacuna* upon Lacuna knockdown **(C)** mRNA levels of *Tbr2* upon Lacuna knockdown **(D)** RNA levels of *Gm33460* upon Lacuna knockdown **(E)** mRNA levels of *Golga4* upon Lacuna knockdown **(F)** Mouse Neural Stem cells were transfected with dCas9-KRAB-mcherry and sgRNA1 targeting *Lacuna* (Lacuna KD-mcherry) or no guide RNA (CTRL-mcherry). They were immunostained for TBR2 (green), mcherry (red) and labeled with DAPI. Scale bar: 100 µm. **(G)** Quantification of TBR2 positive cells in dCas9-KRAB-mcherry positive mouse Neural Stem cells (Control: 72.66 ± 7.624%, Lacuna KD: 47.75 ± 4.825%, p<0.05). **(H)** *Lacuna-GFP* and *Control-GFP* transfected mouse Neural Stem cells were immunostained for Tbr2 (red), GFP (green) and labeled with DAPI. Scale bar: 100 µm. **(I)** Quantification of TBR2 positive cells in transgene positive mouse Neural Stem cells (GFP: 30.43 ± 6.023%, Lacuna: 18.52 ± 3.704%, p>0.05). **(J)** RNA levels of lncRNA *Lacuna* upon *Lacuna* overexpression **(K)** mRNA levels of *Tbr2* upon Lacuna overexpression**.** For all cases, * p<0.05, ** p<0.01, *** p<0.001.

Supplementary Figures Legends

Supplementary Figure 1. *Lacuna (NONMMUT071331.2)* sequence and expression profile from the NONCODE database. (A) *Lacuna* transcript sequence as it was retrieved by the NONCODE Genome Database (http://www.noncode.org/show_rna.php?id=NONMMUT071331&version=2&utd=1#). Note

that *Lacuna* corresponds to the transcript *NONMMUT071331*.2 from the NONCODE. **(B)** *Lacuna* (*NONMMUT071331.2*) was found in the NONCODE Genome Database to be expressed in various adult mouse tissues, including adult mouse hippocampus.

Supplementary Figure 2. Mapping of *Lacuna* locus by RT-PCR assays. (A) Schematic representation of the *Lacuna* gene organization (upper panel) in the mouse genome. The pairs of specific primers that were used to map different exons of *Lacuna* with PCR, are indicated with blue (forward primers) and red (reverse primers) arrows. In the lower panel, the sizes in bp of the expected PCR products are indicated. **(B)** PCR products of each pair were analyzed by agarose gel electrophoresis. PCR template was cDNA (random primers) produced by RNA extracted from mouse embryonic telencephalon of each developmental stage (E12, E14, E16, E18, P0). DNA Ladder: Quick Load Purple 100bp DNA Ladder.

Figure 1.TIF

Figure 1

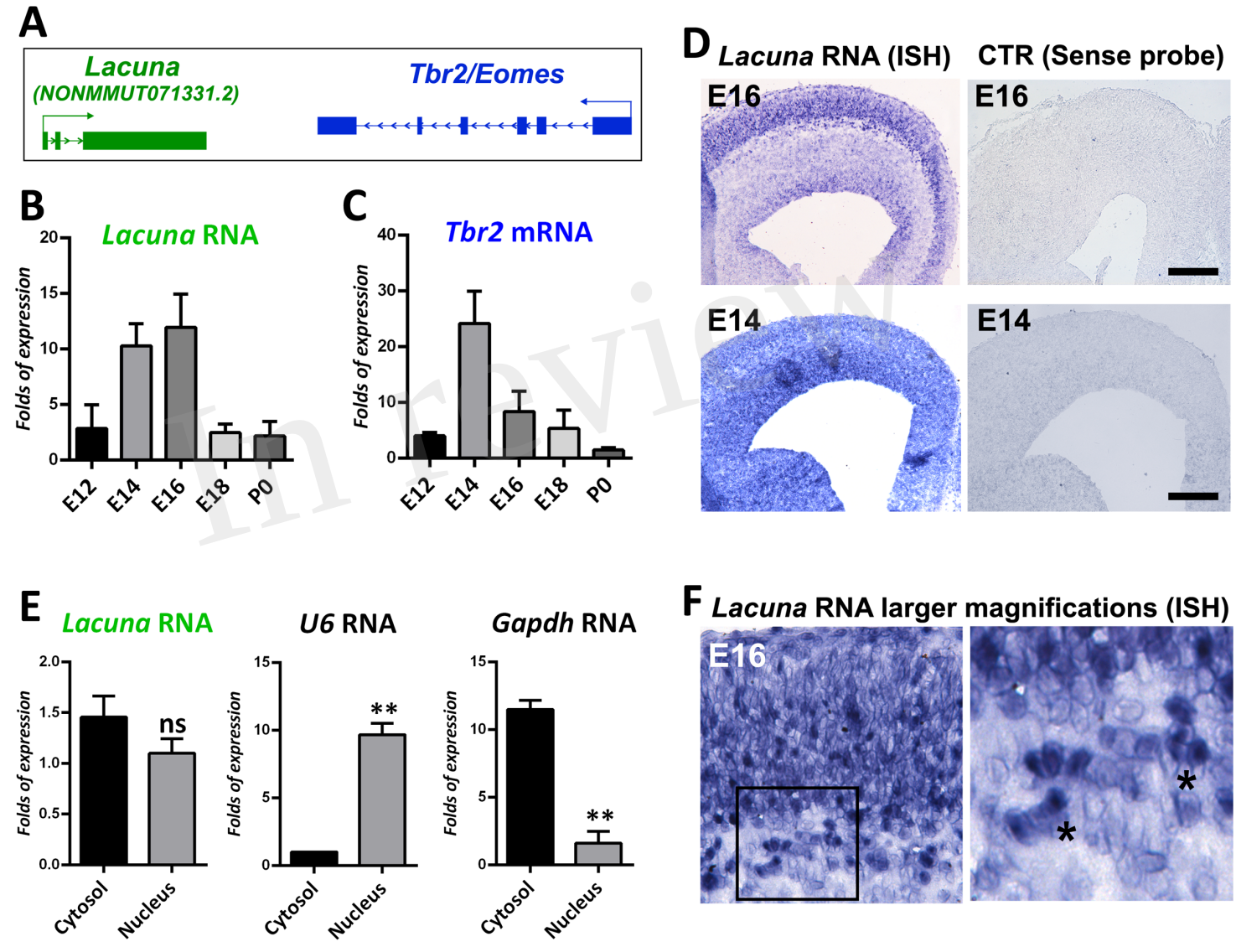
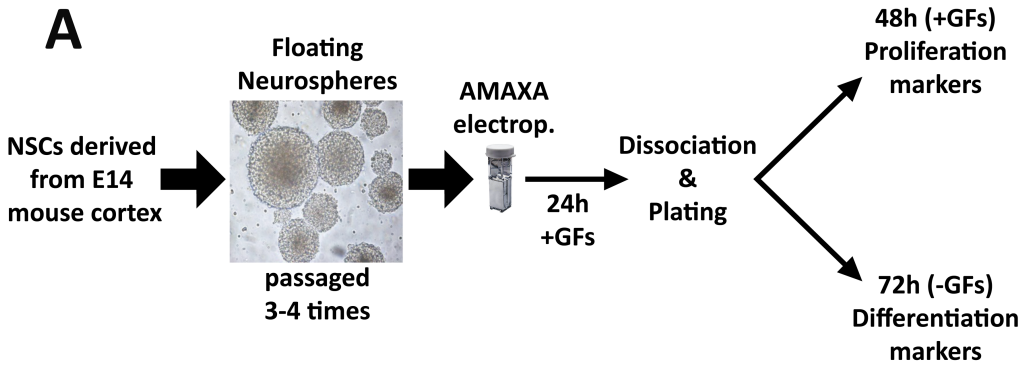
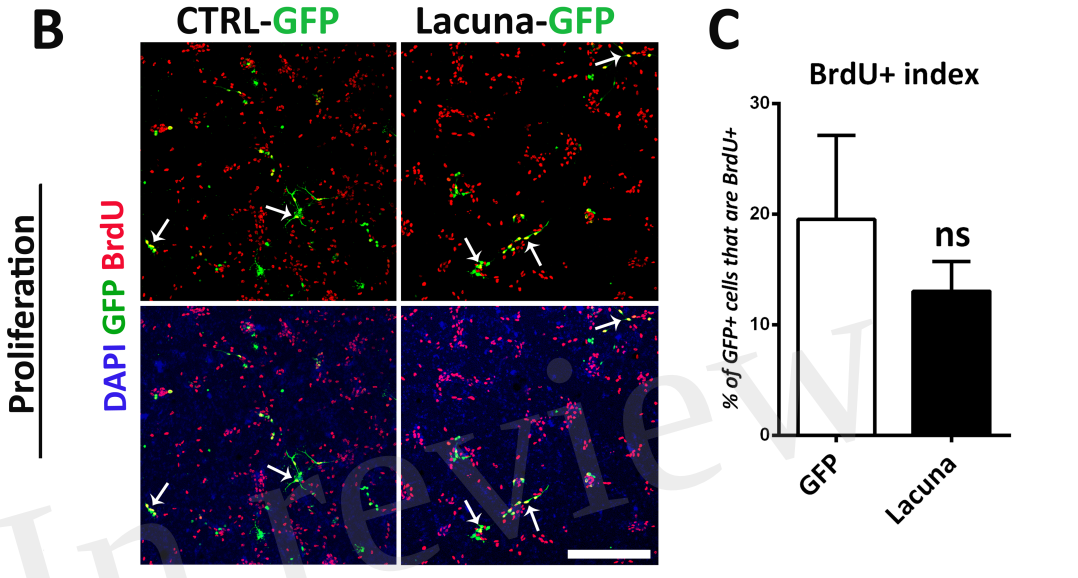
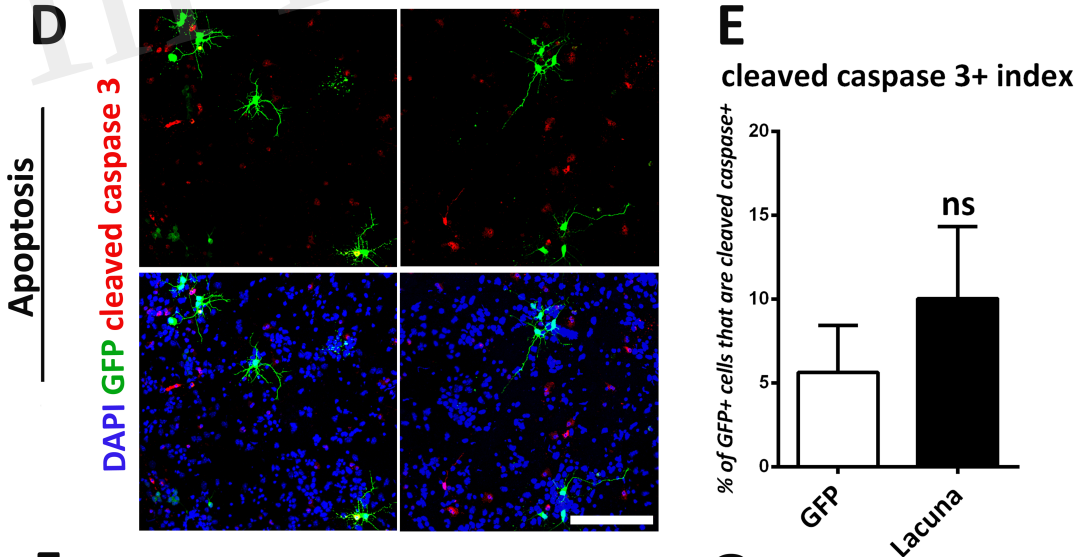


Figure 2

Figure 2.TIF







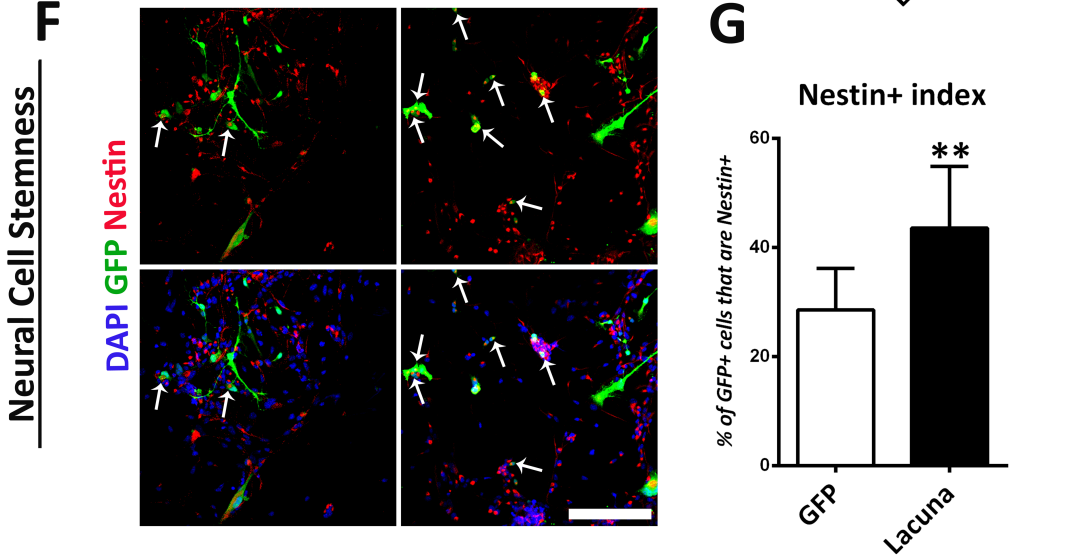


Figure 3

Figure 3.TIF

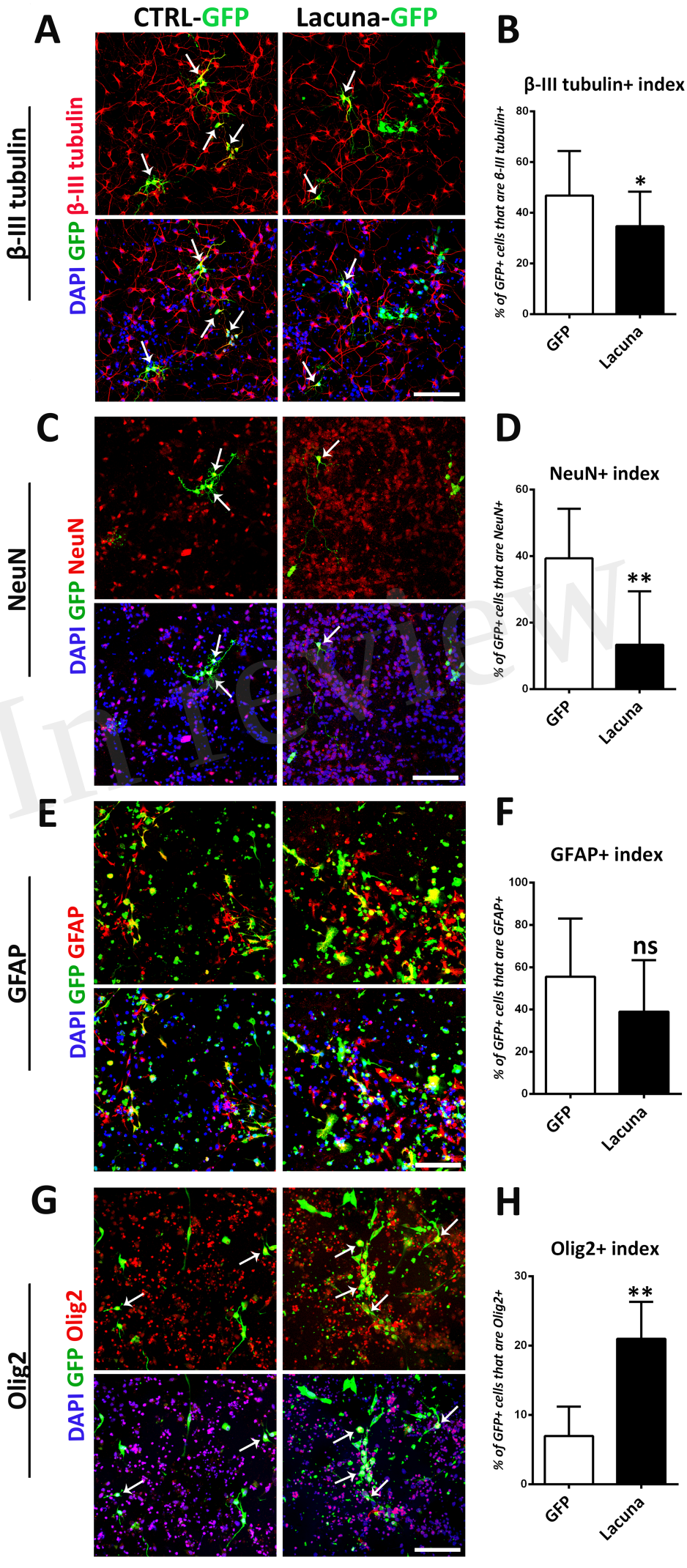


Figure 4

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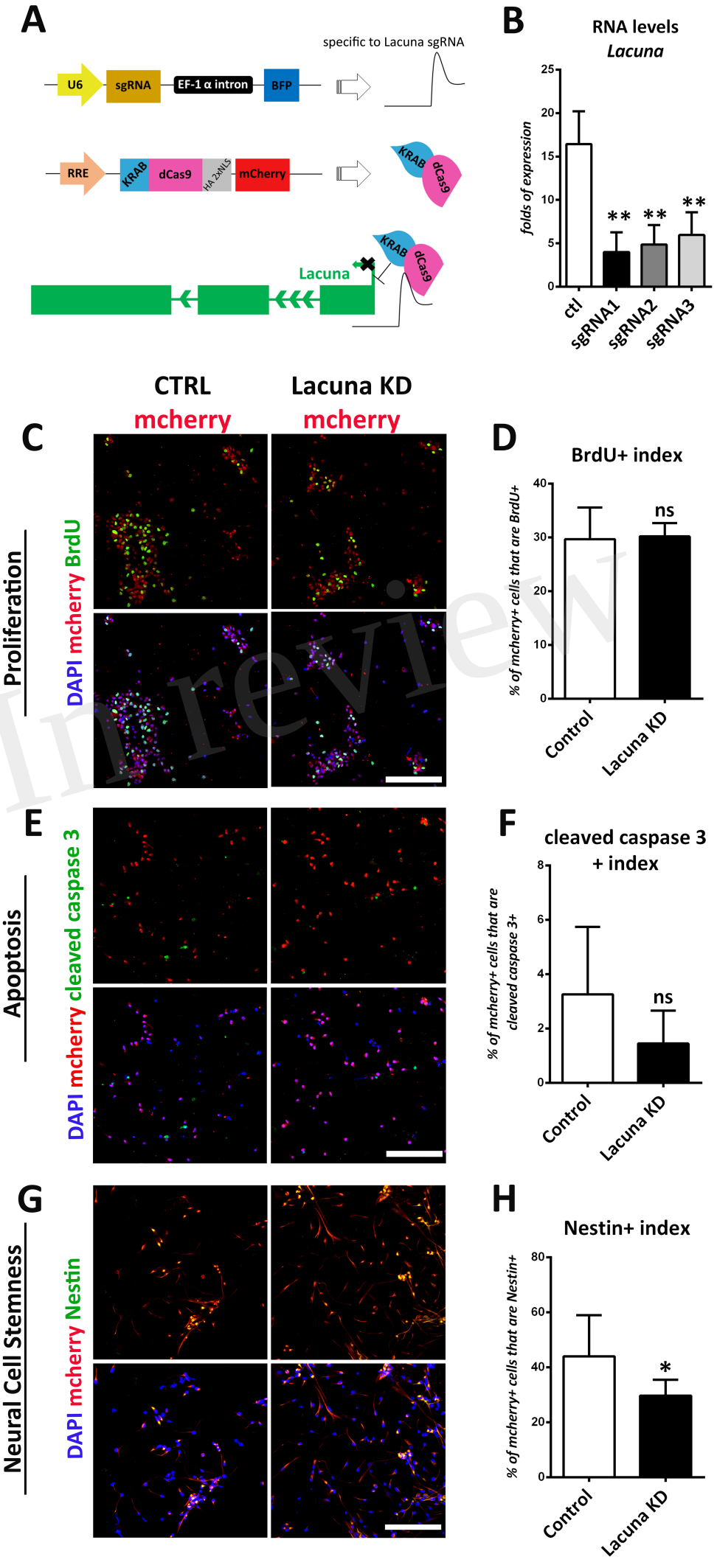

Figure 5

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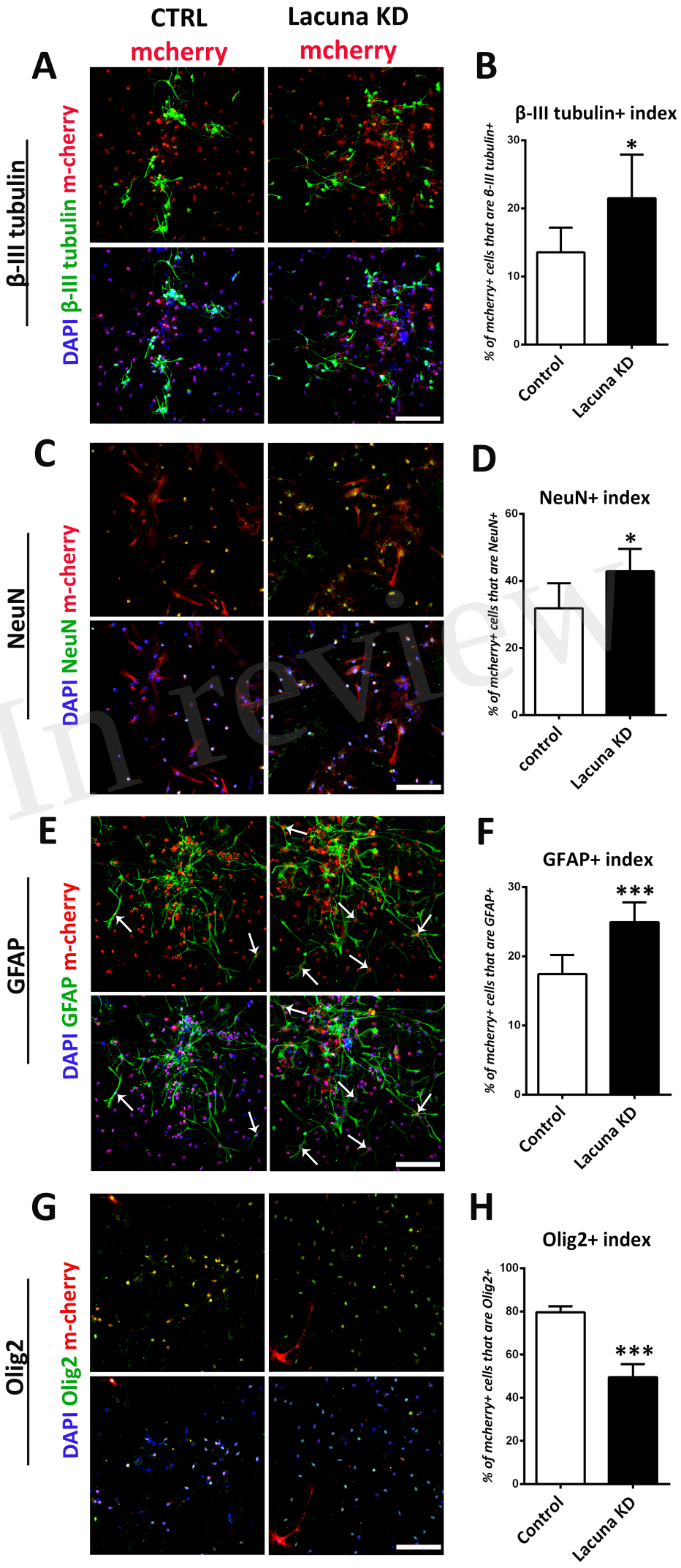

Figure 6.TIF

Figure 6