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Review

Purinosomes and Purine Metabolism in Mammalian Neural Development: A Review

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Neural stem/progenitor cells (NSPCs) in specific brain regions require precisely regulated metabolite production during critical development periods. Purines—vital components of DNA, RNA, and energy carriers like ATP and GTP—are crucial metabolites in brain development. Purine levels are tightly controlled through two pathways: *de novo* synthesis and salvage synthesis. Enzymes driving *de novo* pathway are assembled into a large multienzyme complex termed the "purinosome." Here, we review purine metabolism and purinosomes as spatiotemporal regulators of neural development. Notably, around postnatal day 0 (P0) during mouse cortical development, purine synthesis transitions from the *de novo* pathway to the salvage pathway. Inhibiting the *de novo* pathway affects mTORC1 pathway and leads to specific forebrain malformations. In this review, we also explore the importance of protein-protein interactions of a newly identified NSPC protein—NACHT and WD repeat domain-containing 1 (Nwd1)—in purinosome formation. Reduced Nwd1 expression disrupts purinosome formation, impacting NSPC proliferation and neuronal migration, resulting in periventricular heterotopia. Nwd1 interacts directly with phosphoribosylaminoimidazole–succinocarboxamide synthetase (PAICS), an enzyme involved in *de novo* purine synthesis. We anticipate this review will be valuable for researchers investigating neural development, purine metabolism, and protein-protein interactions.

Key words: neural stem/progenitor cells (NSPCs), purine metabolism, purinosome, Nwd1, neural development

I. Purine Metabolism

Purines (compounds containing a pyrimidine ring fused with an imidazole ring) are found in all living organisms. They play essential roles as the building blocks of DNA and RNA, especially adenine and guanine, the energy molecules such as ATP and GTP, and the second messengers in both intracellular and extracellular signaling pathways such as cyclic AMP, cyclic GMP, adenosine and ATP [69]. Additionally, adenosine and ATP participate in extracellular signaling through purinergic receptors, which regulate various cell functions such as cell migration, apoptosis, proliferation, and differentiation [34, 43]. Figure 1 provides an overview of purine metabolism, illustrating the metabolites, enzymes, and chemical inhibitors. There are two pathways for purine synthesis in mammals: 1) the *de novo* purine synthesis and 2) the salvage purine synthesis (henceforth referred to as the *de novo* pathway and salvage pathway). In the salvage pathway, purines are synthesized by reusing degraded bases, including AMP, GMP, and IMP obtained from catabolism [36]. The enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT or HPRT) con-

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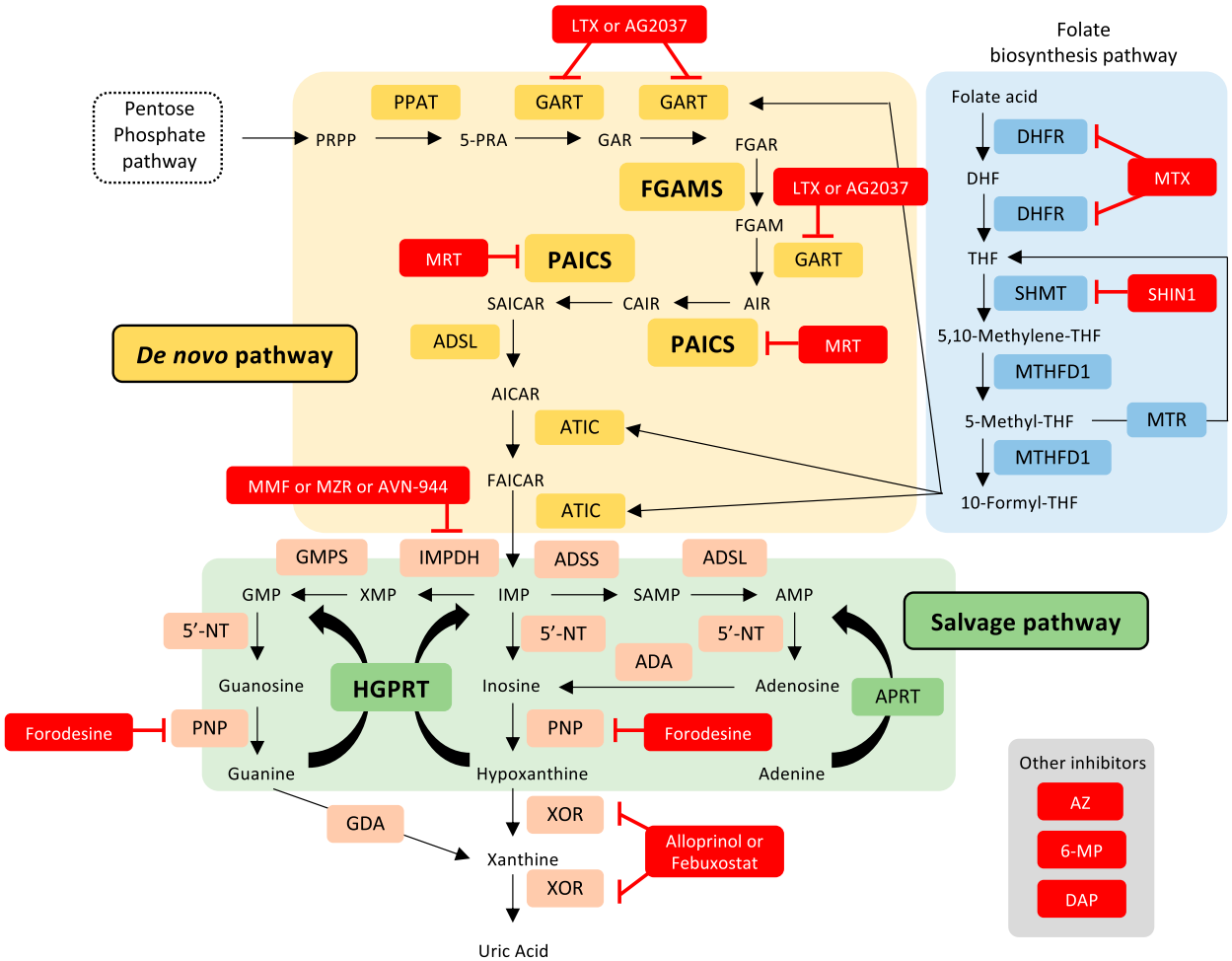


Fig. 1. *De novo* and salvage purine synthesis pathways in mammals [50]. A schematic diagram illustrating metabolic enzymes participating in the *de novo* and salvage pathways, represented by yellow and green boxes, respectively. The blue box illustrates the folate biosynthesis pathway, crucial for producing substrates required for *de novo* purine synthesis; Chemical inhibitors are highlighted in red [purine analogs: 6-Mercaptopurine (6-MP) and azathioprine (AZ), AZ is metabolized to 6-MP [28, 47]; 2,6-diaminopurine (DAP); analog of adenine [6], lometrexol (LTX) and pelitrexol (AG2037); GART inhibitors [21, 33], MRT00252040 (MRT); PAICS inhibitor [33], mycophenolate mofetil (MMF), mizoribine (MZR), and AVN-944; IMPDH inhibitors [1, 21, 52], forodesine hydrochloride (Forodesine); PNP inhibitor [24], allopurinol and febuxostat; XO inhibitors [53, 65], methotrexate (MTX); DHFR inhibitor [16]; SHMT inhibitor1 (SHIN1) [46].

verts hypoxanthine and guanine to IMP and GMP, respectively, while adenine phosphoribosyltransferase (APRT) synthesizes AMP from adenine (Fig. 1) [56]. Under normal conditions, purines are predominantly supplied through the salvage pathway. However, the *de novo* pathway is activated when cellular demand for purines exceeds the capacity of salvage synthesis, particularly in situations that require higher levels of purines and their derivative nucleotides, such as during tumor growth and cell proliferation [55, 82].

The *de novo* pathway generates IMP from 5-phosphoribosyl-1-pyrophosphate (PRPP)—which is formed within the glycolysis-pentose phosphate pathway—through ten sequential reactions mediated by six enzymes [55, 56]. The six enzymes are: 1) phosphoribosyl pyrophosphate amidotransferase (PPAT); 2) glycinamide ribonucleotide transformylase (GART); 3) formylglycinamidine ribonucleotide synthase (FGAMS); 4) phosphoribosylaminoimidazole–succinocarboxamide synthetase (PAICS); 5) adenylosuccinate lyase (ADSL); and 6) 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (ATIC) (Fig. 1). In addition to PRPP, aspartate from the tricarboxylic acid cycle, and glycine and formate from the folate biosynthesis pathway are required to drive the *de novo* pathway [55]. Formate—generated by the mitochondrial enzymes dihydrofolate reductase (DHFR), Serine hydroxymethyltransferase-2 (SHMT2), and methylenetetrahydrofolate dehydrogenase (MTHFD) as part of one-carbon metabolism—is converted to 10-Formyltetrahydrofolate (10-formyl-THF) and integrated into the *de novo* pathway through cytosolic enzymes GART and ATIC (Fig. 1) [19, 38, 55, 84].

II. Purinosome

In 2008, a significant advancement in purine metabolism was made with the discovery of the purinosome. The purinosome is a complex of six *de novo* pathway enzymes (PPAT, GART, FGAMS, PAICS, ADSL and ATIC). Assembled as a large multi-complex structure, it boosts the efficiency of *de novo* purine synthesis, particularly in cancer cells (Fig. 2A, B) [2, 56]. The purinosome is a metabolon, a complex structure comprising sequential metabolic enzymes [56]. Additionally, the purinosome contains several chaperones, including HSP70, HSP90, DnaJA1, and DnaJc7 [25, 57].

The molecular triggers for purinosome formation are not fully understood. The first three enzymes in the *de novo* pathway (tetrameric PPAT, monomeric FGAMS, and dimeric GART) form the purinosome core; the octameric PAICS, tetrameric ADSL, and dimeric ATIC, subsequently, interact with this core [29, 42, 56, 70]. By spatially organizing these enzymes, the purinosome minimizes the diffusion of intermediates and facilitates the rapid and coordinated synthesis of purine nucleotides. Pareek *et al.* demonstrated this through *in situ* three-dimensional sub-micrometer chemical imaging of single HeLa cells using gas cluster ion beam secondary ion mass spectrometry (GCIB-SIMS). They revealed that the purinosome is not merely a protein aggregate. It activates *de novo* purine synthesis, thus promoting the production of purine metabolites [55]. Furthermore, a recent study identified endogenous purinosome puncta using PAICS-GFP knock-in HeLa cells [12]. To ensure efficient purine metabolite production, purinosomes tend to form near the mitochondria, presumably to utilize the mitochondrial metabolites produced in the folate biosynthesis pathway [26]. In addition, timelapse imaging showed that purinosomes exhibit directed transport; they move along microtubules towards the mitochondria [3, 11].

Purinosome formation is regulated by various cellular signals and conditions, including substrate availability, energy status, and the cell cycle stage. Notably, purinosome formation is highest during the G_1 phase of the cell cycle [10]. Cellular signaling pathways, such as those involving AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR), can influence purinosome assembly in response to changes in cellular energy levels [26, 45, 63]. Purinosomes can be induced to form *in vitro* by a) purine depletion using dialyzed fetal bovine serum (FBS) [2]; b) casein kinase II (CK2) inhibition using inhibitors such as DMAT and TBI [4]; c) stimulation of G protein-coupled receptors (GPCRs) with agonists like oxymetazoline [71]; d) disrupting salvage pathway (HGPRT knockdown) [27]; e) causing mitochondrial dysfunction using antimycin A and oligomycin [26]; f) inducing hypoxia [17]; and g) accelerating liquid-liquid phase separation (Fig. 2C) [12, 58].

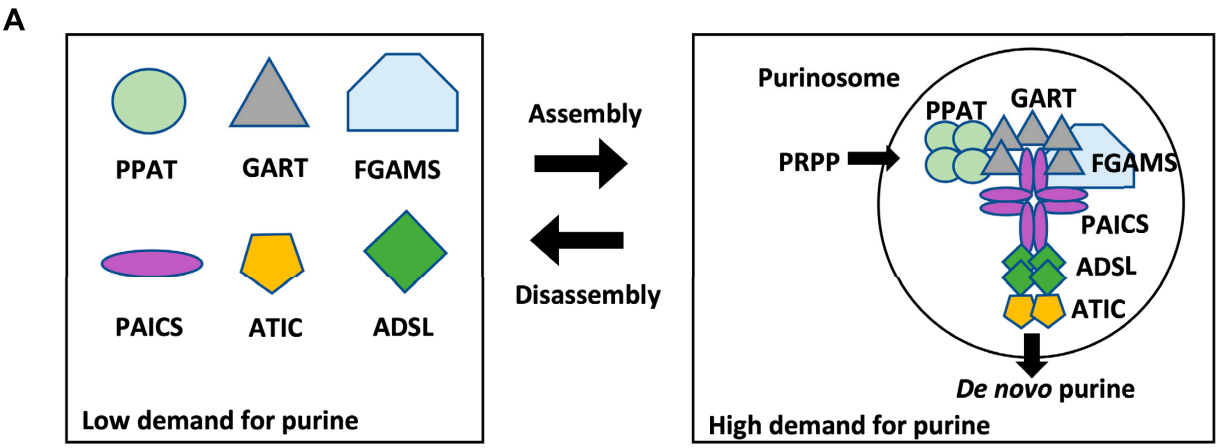
III. Neural Development

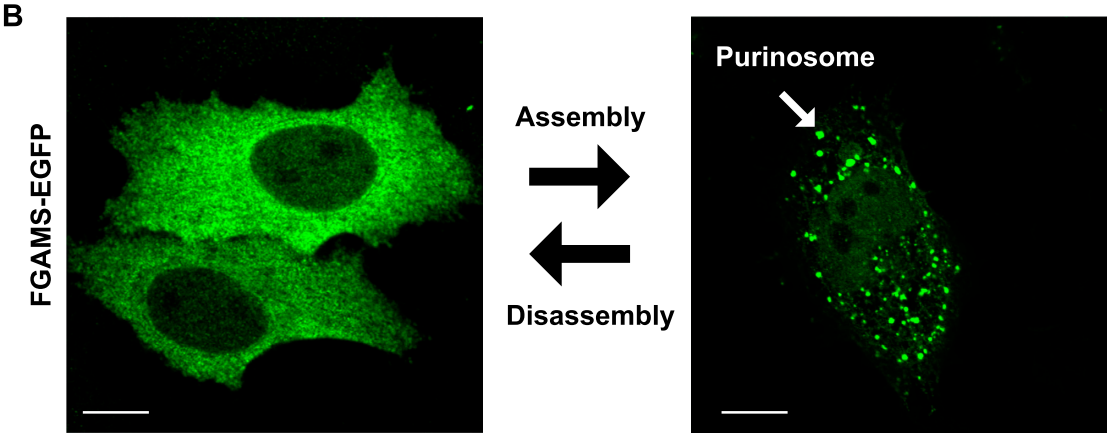
The spatiotemporal balance of proliferation and differentiation of neural stem/progenitor cells (NSPCs) are crucial for the normal central nervous system (CNS) development. In the developing cerebral cortex of mammalian embryo, two distinct types of NSPCs are present: 1) the apical progenitors (APs) (also called apical radial glia) located in the apical-most part of the neocortex known as the ventricular zone, and 2) the basal progenitors (BPs) generated by APs, located in the subventricular zone. BPs consist of two major subtypes: basal intermediate progenitors (bIPs) and basal (outer) radial glia (bRG) [23, 31]. Primates, including humans, have abundant bRG, whereas rodents rarely have this subtype [31]. Newborn neurons generated from NSPCs in the ventricular and subventricular zones migrate radially toward the cortical plate and undergo sequential changes in cell shape. Defects in the proliferation of NPSCs and neuronal migration can lead to brain malformations such as microcephaly, lissencephaly, and periventricular heterotopia (PH), as well as contribute to various psychiatric disorders, including epilepsy and mental retardation [30, 61].

IV. Purine Metabolism in Neural Development

Unraveling purine metabolism is crucial for understanding brain development and its related abnormalities. In humans, several neurological pathologies are linked to purine metabolism. Inherited deficiencies in *de novo* pathway enzymes often lead to fetal lethality or neurological diseases [36]. For instance, a missense mutation in PAICS causes multiple physical malformations, including a small body, short neck, and craniofacial dysmorphism, and causes early neonatal death [59]. Moreover, deficiencies in ADSL or ATIC result in severe developmental brain defects such as mental retardation, autism, epilepsy, microcephaly, congenital blindness, speech impairment, and auto-aggressive behavior [20, 37, 49]. Similarly, prenatal exposure to methotrexate (MTX), which inhibits DHFR (a folate biosynthesis pathway enzyme) (Fig. 1), leads to fetal death. Moreover, children who survive this treatment, often exhibit cranial dysostosis, dysmorphic facial features, skeletal malformations, limb defects, growth retardation, and developmental delay; and they also exhibit severe CNS anomalies including semilobar holoprosencephaly (HPE), holoprosencephaly, and other brain malformations [13, 64]. Salvage pathway deficiencies, such as HGPRT deficiency, lead to Lesch–Nyhan syndrome, characterized by juvenile gout, dystonia, mental retardation, and compulsive self-injurious behavior [41, 68]. Previous studies of human pathologies strongly suggest the importance of a balance between *de novo* and salvage purine synthesis pathways for healthy brain development.

Our recent study delved into the expression profile and functional significance of purine synthesis enzymes in the





C

Induction of purinosome assembly

• Purine depletion Example: Dialyzed FBS
• CK2 inhibitor Example: DMAT, TBI
• GPCR agonist Example: Oxymetazoline
• Purine salvage (HGPRT) deficiency
• Disruption of mitochondrial processes Example: Antimycin A, Oligomycin
• Hypoxia
• Liquid-liquid phase separation

Fig. 2. Summary of purinosome, a metabolon involved in purine metabolism. (**A**) Hypothetical model of purinosome formation; (**B**) Representative images of FGAMS-EGFP^{+} purinosome assembly in HeLa cells [Bar = 10 μm]; (**C**) Methods of purinosome assembly: Purine depletion assay using dialyzed FBS [2]; casein kinase II (CK2) inhibition using DMAT and TBI [4]; GPCR inhibition using GPCR agonist *i.e.* oxymetazoline [71]; inducing purine salvage (HGPRT) deficiency [27]; inducing mitochondrial dysfunction by antimycin A and oligomycin [26]; inducing hypoxia [17]; accelerated liquid-liquid phase separation [12, 58].

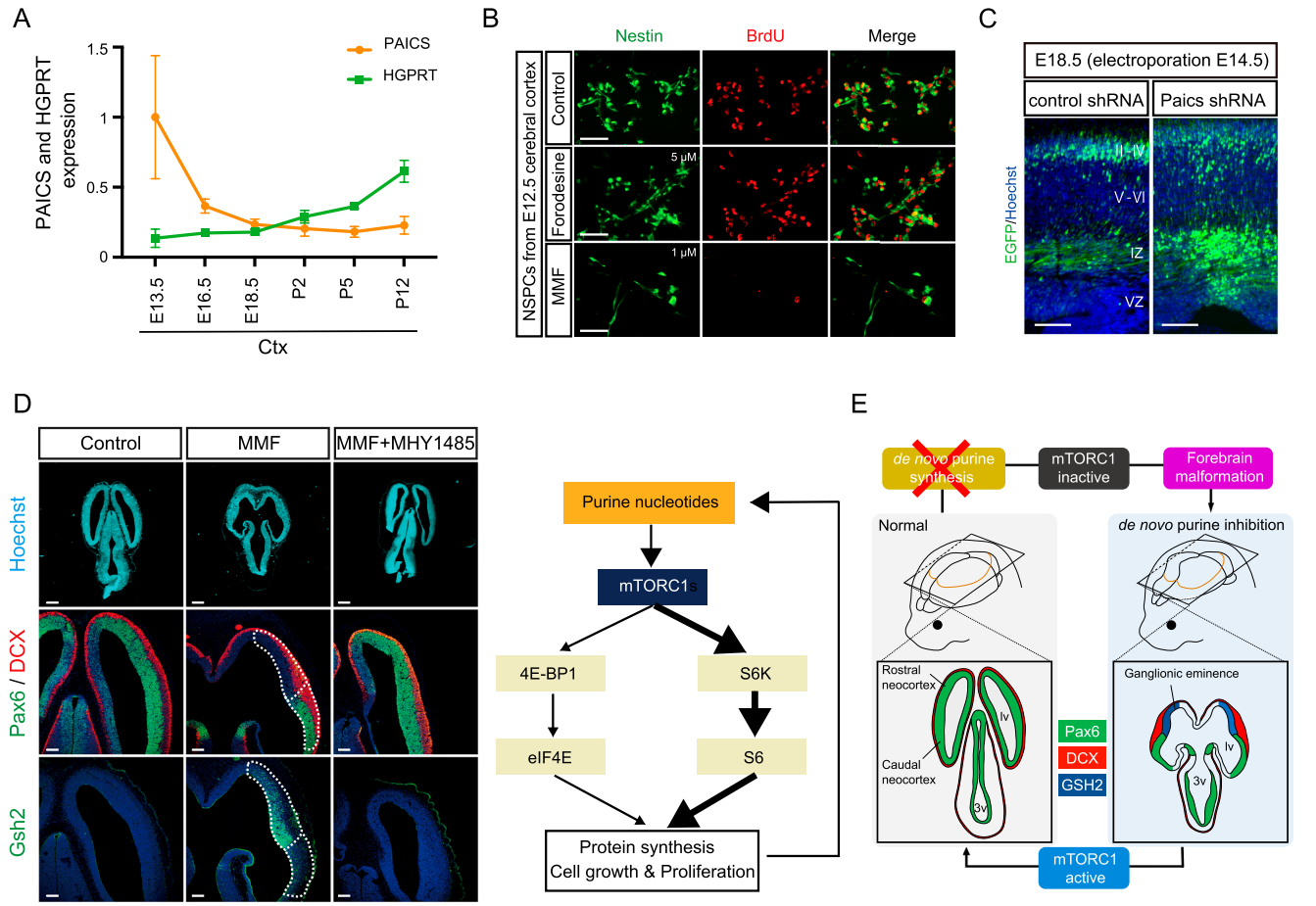


Fig. 3. Spatiotemporal regulation of *de novo* and salvage purine synthesis pathways in mammalian brain development [50, 80]. (**A**) The expression of PAICS (*de novo* pathway enzyme) and HGPRT (salvage pathway enzyme) shift during cortical development at neonatal period (P0); (**B**) Inhibition of *de novo* pathway disturbs NSPC proliferation *in vitro*. Primary cultured NSPCs were treated with forodesine or MMF for 48 hr, followed by BrdU labeling for 24 hr. NSPCs were then immunostained with anti-Nestin and anti-BrdU antibodies; (**C**) Inhibition of PAICS by *in utero* electroporation at E14.5 decreased NSPCs population and caused defects in neuronal migration during mice brain development; (**D**) Inhibition of *de novo* pathway caused forebrain-specific malformation through regulation of mTORC1/S6K-S6 pathway. Pregnant mice were administered MMF and MHY1485 between E9.5–11.5 and analyzed at E12.5. (Left panels) Representative images of horizontal sections immunostained with anti-Pax6, anti-DCX, and anti-Gsh2 antibodies; (Right panel) A schematic diagram illustrating the relationship between the mTOR signal cascade and purine nucleotides; (**E**) A schematic model depicting brain malformations resulting from inhibiting *de novo* pathway. MMF-administrated brains show a hypoplasia of the rostral neocortex and an expansion of the dorsal Gsh2$^+$ striatal ganglionic eminence (blue). The rostral neocortex with Pax6$^+$ ventricular zone disappeared, replaced by the ganglionic eminence with a Gsh2$^+$ ventricular zone ectopically appearing on the dorsal surface [IZ, intermediate zone; VZ, ventricular zone; lv, lateral ventricles; 3v, third ventricle].

developing mammalian brain. Each purine pathway exhibits distinct temporal and regional characteristics during brain development [50]. We revealed that purine synthesis transitions from the *de novo* to the salvage pathway in the neonatal period (Fig. 3A) [50]. FGMAS and PAICS, *de novo* pathway enzymes, are expressed abundantly in the early embryonic stages, while HGPRT expression is upregulated in the postnatal period (Fig. 3A). Hypoxanthine, a salvage pathway metabolite, influences morphologic changes in microglia [54]. Microglia likely represent the primary cell type utilizing the salvage pathway from late embryonic to postnatal stages. Immunohistochemistry—using anti-FGAMS, anti-PAICS, and anti-HGRPT antibodies in developing and adult mice brains—revealed that utilization of purine synthesis pathways during cerebral and cerebellar cortex development varies according to developmental stage and is region-specific [50]. Ultrastructural analysis using immunoelectron microscopy in primary cultured rat hippocampal neurons revealed that FGAMS, PAICS, and ATIC are localized in the mitochondria or near it [76]. *In vitro* study demonstrated that MMF (a *de novo* GMP synthesis inhibitor) but not forodesine (a salvage pathway inhibitor) significantly reduced NSPC proliferation and induced apoptosis (Fig. 3B) [50]. Similarly, multipotent NSPC proliferation relies solely on the active *de novo* pathway, whereas glial cell proliferation relies on either *de novo* or salvage pathways [50, 62]. Purinergic signaling is essential for NSPC maintenance and neuronal

migration in the neocortical subventricular zone during brain development [43, 44]. In addition, purine metabolites, including ATP and GTP/GDP, are critical for polarity formation in postmitotic cortical neurons [60]. We found that PAICS knockdown (via *in utero* electroporation) reduces the NSPCs population and causes defects in neuronal migration during embryonic cortical development (Fig. 3C) [80]. Other studies using ADSL-knockdown chicken and zebrafish embryos revealed impaired neurogenesis and microcephaly accompanied by defective ciliogenesis [20]. Thus, both—reduced purine levels and impaired *de novo* purine synthesis—contribute to purine-related neurodevelopmental pathologies.

Through *in vivo* pharmacological studies using different inhibitors of purine synthesis, we confirmed that the spatiotemporal regulation of these two purine synthesis pathways is essential for normal mouse brain development. Experiments with mice treated with MMF, MTX, or forodesine showed that both pathways cooperatively regulate cerebellar development [41]. On the other hand, inhibitors targeting *de novo* pathway exclusively suppressed NSPC proliferation and delayed neuronal migration in the developing cerebral cortex [50]. Intriguingly, embryos continuously exposed to MMF exhibit severe forebrain cortical malformations, suggesting a gradient of purine demand along the anteroposterior axis of the embryonic brain, with higher requirements in the dorsal forebrain cortical areas than in the ventral or posterior areas (Fig. 3D, E) [50]. MMF-treated rostral neocortex expressed glutathione synthetase 2 (Gsh2–a marker of ganglionic eminence) but not paired box protein Pax6, suggesting that rostral neocortex is derived from the lateral ganglionic eminence (Fig. 3D, E) [50]. In addition to treatment with MMF, we showed that the treatments with MTX and LTX also induce severe brain malformations and neural tube defects [50, 73, 78].

De novo purine synthesis is also important for the proper functioning of the mature brain. FGAMS is weakly expressed in the adult brain [48, 50, 76]. Correspondingly, continuous MTX administration to mice (juvenile-age onwards) reduces adult neurogenesis in the hippocampus and causes deficits in spatial memory and visual recognition memory [74, 75, 83]. These findings highlight the continued requirement of *de novo* purine synthesis in specific brain regions and cell types even into adulthood. Thus, these findings contribute to our understanding of neurological disorders linked to *de novo* pathway defects.

In mammalian cultured cells, purine production is closely related to the mammalian/mechanistic target of the rapamycin complex 1 (mTORC1) signaling pathway and the expressions of eukaryotic initiation factor 4E (eIF4E)/binding protein 1 (4E-BP1) and ribosomal protein S6 kinase (S6K)/S6 proteins [8, 21, 33, 51]. Mutations in mTORC1 affect cerebrum development, suggesting its role in NSPCs development [5, 67]. We demonstrated that the administration of MHY1485 (an mTOR signal activator) [54] restores the MMF-induced cortical malformation [50]. Thus, the *de novo* pathway is strongly associated with mTORC1/S6K-S6 signaling cascade and may be related to mTORC1/4EBP1/eIF4E signaling cascade during cortical development [50].

Notably, the effects of deficiencies in the salvage and *de novo* pathways are different. HGPRT deficiency primarily leads to functional impairment of the dopamine system in the basal ganglia, while structural brain abnormalities are absent [32, 39, 72, 77]. Double knockout mice lacking both APRT and HGPRT (i.e., totally lacking salvage pathway) do not exhibit behavioral abnormalities related to Lesch–Nyhan syndrome [22]. Similarly, administration of forodesine to embryonic mice did not affect cortical development [50]. Thus, HGPRT deficiency mediates functional deficits in the dopamine system resulting from increased cell division and altered migration patterns of the midbrain dopamine neurons during early embryonic stages [77]. These *in vivo* findings might directly evidence the neurodevelopmental signature of Lesch–Nyhan syndrome. *In vitro* omics study using iPS lines from individuals with Lesch–Nyhan syndrome showed reductions in *FAR2P1* mRNA, and NSEA4 and EID3 proteins, although the underlying reasons for these changes remain unclear [66]. Furthermore, cultured human NSPCs isolated from fetal brains of individuals with Lesch–Nyhan syndrome exhibited decreased FLT4 and β-tubulin III expression and impaired neuronal differentiation [15]. Further research is necessary to elucidate the molecular mechanisms of Lesch–Nyhan syndrome and to comprehensively interpret the *in vitro* and *in vivo* phenotypes associated with this syndrome.

V. Nwd1 in Brain Development

Previously, we employed suppression subtractive hybridization technique to identify novel genes highly expressed solely in NSPCs (Fig. 4A) [35, 79, 85]. This approach led us to discover the differentially expressed *NACHT and WD repeat domain-containing protein 1* (*Nwd1*) gene; we confirmed robust expression of its transcript and protein in NSPCs during embryonic mouse brain development [79]. Nwd1 localizes in the mitochondria and endoplasmic reticulum [79, 81]. To elucidate the physiological roles of Nwd1, we suppressed Nwd1 expression (using *in utero* electroporation of *Nwd1* shRNA) in the embryonic mouse cerebral cortex. Nwd1 knockdown resulted in defects in the $Ki67^+$ proliferating NSPCs in the ventricular zone (Fig. 4B), premature differentiation into immature neurons, and delayed neuronal migration [80]. Notably, postnatal day 7 (P7) pups with suppressed Nwd1 expression at E14.5 exhibit PH, a developmental cortical dysgenesis frequently characterized by focal drug-resistant epilepsy in humans (Fig. 4C, D, Arrows) [7, 80]. PH is usually associated with an excessive formation of neural circuits by $VgluT1^+$ excitatory glutamatergic neurons (Fig. 4E) [80].

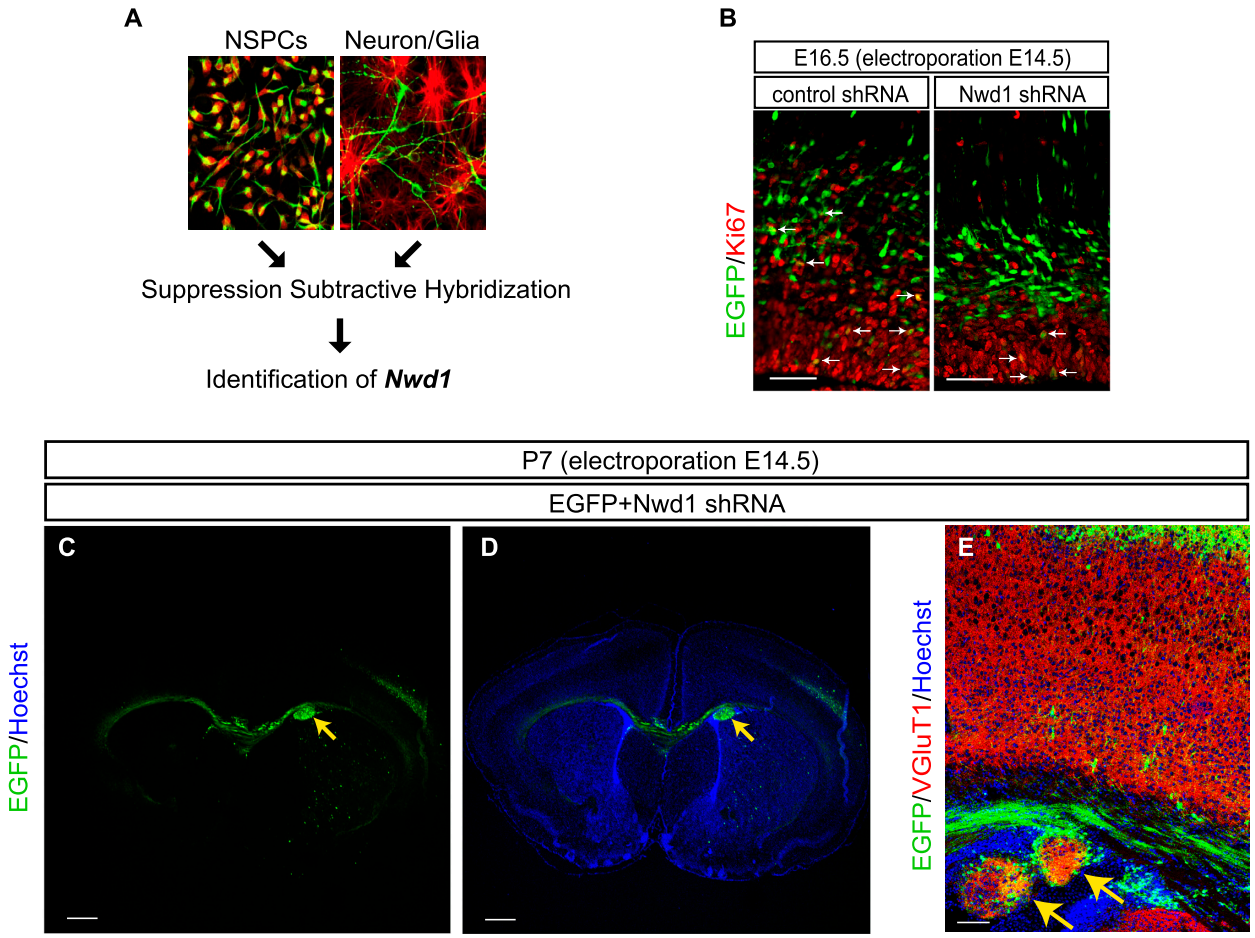


Fig. 4. Identification and functional analysis of Nwd1 in cortical development [79, 80]. (**A**) *Nwd1* gene was identified by comparing gene expression patterns in primary cultured NSPCs and differentiated cells, including neurons and glial cells, using the suppression subtractive hybridization technique; (**B**) Knockdown of *Nwd1* gene by *in utero* electroporation of shRNA at E14.5 reduced proliferation of Ki67^{+} NSPCs during mouse cortical development; (**C**–**E**) *Nwd1* shRNA was introduced into the fetal brain (E14.5) by *in utero* electroporation, and brain development was analyzed at postnatal day 7 (P7). Nwd1 knockdown cells aggregated in the ventricular and subventricular zone, resulting in PH (yellow arrows); (**E**) PH formation was associated with the expansion of VGluT1^{+} glutamatergic neurons [Bars = 50 μm in (**B**); 500 μm in (**C**)–(**D**); and 100 μm in (**E**)].

VI. Nwd1 in Purinosome Assembly

The Nwd1 protein comprises a central NACHT domain (predicted to possess nucleoside-triphosphatase (NTPase) activity) and a cluster of WD40 repeats at the C-terminus [79]. Based on its molecular architecture, Nwd1 is classified as a member of the signal transduction ATPases with numerous domains (STAND) protein superfamily [79]. Other members of the STAND protein family often mediate ligand-induced self-oligomerization to form large multiprotein complexes such as the "apoptosome" and "inflammasome"; these complexes are induced by the apoptotic peptidase activating factor 1 (Apaf1) and nucleotide-binding oligomerization domain-like receptors (NLRs), respectively [9, 18, 40]. Notably, Nwd1 and Apaf1 have structurally analogous domains [18, 79]. While uncovering how Nwd1 regulates corticogenesis, we hypothesized that the N-terminal region of Nwd1 serves as an effector domain, enabling the protein to bind signaling molecules and trigger self-oligomerization mediated by its NACHT domain and WD40 repeats. Therefore, we sought to identify the Nwd1 binding partner, with a special focus on its N-terminal effector domain.

Various methodologies exist for studying protein-protein interaction (outlined in Table 1), each with its own strengths and limitations. The choice of method depends on specific research needs, including a) the type of interaction being studied, b) required sensitivity and specificity, c) whether the study is *in vitro* or *in vivo*, and 4) the available equipment and expertise. Among the available methods, we employed yeast-two-hybrid (Y2H) screening (TAKARA Bio) as a potent tool for identifying the unknown Nwd1 direct binding proteins (Fig. 5A) [80]. This approach led us to identify PAICS as the binding partner of the Nwd1-N terminal domain [80]. Further, we screened for proteins interacting with full-length Nwd1. Attempts to purify

Table 1. *Methods for detecting protein-protein interactions*

	Strengths	Limitations
・His-/GST-/MBP-/Halo-tag binding assay using E. coli and LC-MS/MS	・Low-cost ・High-purified-volume ・High specificity of purification ・Can be identified direct binding protein	・Cannot be expressed over 100 kDa protein ・Easily insoluble ・May not reflect native interactions
・Yeast-two-hybrid assay using Yeast	・Can be identified direct binding proteins ・Effective for screening	・Cannot be expressed over 100 kDa protein ・Can be detected some false positives
・Co-immunoprecipitation using mammalian cells and LC-MS/MS (antibody dependent)	・Any protein size ・Can be identified endogenously interacting protein	・Can be detected indirectly interaction ・Dependent of antibody specification
・Halo-tag binding assay using mammalian cells and LC-MS/MS (antibody independent)	・Any protein size ・Easily soluble ・High specificity of purification	・Can be detected indirectly interaction ・low-purified-volume
・FRET using mammalian cells and microscope	・Can be observed direct binding proteins ・High spatiotemporal resolution	・Cannot be used for screening ・Need some specific equipment
・Split fluorescent proteins using mammalian cells and microscope	・Can be observed direct protein binding ・High spatial resolution	・Cannot be used for screening
・Proximity ligation assay using mammalian cells and microscope	・Can be observed direct protein binding ・High spatial resolution	・Cannot be used for screening

recombinant His- or GST-tagged Nwd1 protein using the bacterial expression system were unsuccessful due to insolubility issues (Fig. 5A). However, the Halo-tag-based binding assay using HEK293T cells and proteome analysis using LC-MS/MS proved effective in screening proteins interacting with full-length Nwd1 (Fig. 5A) [81]. A previous study expressing Flag-Nwd1 in LNCaP prostate cancer cell line identified multiple chaperones (such as HSP70 and HSP90) as full-length Nwd1 binding partners through co-immunoprecipitation and proteome analysis (Fig. 5A) [14].

Co-immunoprecipitation and immunostaining analysis also revealed Nwd1 as a novel member of purinosome alongside FGAMS and PAICS in HeLa cells (Fig. 5B) [80]. We showed that FGAMS and PAICS co-localize as granular structures in primary cultured NSPCs, indicating that purinosomes form within cellular processes and cell bodies even under normal physiological conditions (Fig. 5C) [80]. Our study was the first to evidence purinosome assembly under normal physiological conditions. Silencing the Nwd1 expression in primary cultured NSPCs reduces the formation of $FGAMS^+$ and $PAICS^+$ purinosomes, indicating that Nwd1 is involved in purinosome formation in NSPCs (Fig. 5D) [80]. In summary, our findings suggest that the dynamic assembly of purinosomes through the Nwd1-PAICS interaction is crucial for proper mammalian brain development (Fig. 5E).

Given that purinosomes are abundantly present in two cell types—tumor cells and NSPCs—with high purine demand, it is reasonable to assume that purinosomes also play a crucial role in stem cells of other tissues. However, direct evidence for *in vivo* localization of purinosome in various organs is currently lacking. Therefore, detecting purinosome formation and analyzing its functions *in vivo* will be crucial for future research. Moreover, the molecular factors responsible for the *de novo* to salvage pathway switch, and how *de novo* pathway inhibitors cause brain malformations, remain unclear. Future studies must focus on regional and lineage-specific variations in purine metabolism, especially in purine metabolites and related transcription factors in the brain. They will enhance our understanding of diseases resulting from abnormalities in purine metabolism, including defective purinosome formation. Such studies will also contribute to the development of anticancer medications which target purinosomes.

VII. Abbreviations

Metabolites: PRPP, 5-phosphoribosyl-1-pyrophosphate; 5-PRA, 5-phosphoribosylamine; GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; FGAM, formylglycinamidine ribonucleotide; AIR, aminoimidazole ribonucleotide; CAIR, 4-carboxy-5-aminoimidazole ribonucleotide; SAICAR, 4-(N-succinylcarboxamide) 5-aminoimidazole ribonucleotide; AICAR, aminoimidazole-4-carboxamide ribonucleotide; FAICAR, 5-formylaminoimidazole-4-carboxamide ribonucleotide; IMP, inosine monophosphate; XMP, xanthosine monophosphate; GMP, guanosine monophosphate; SAMP, adenylosuccinate; AMP, adenosine monophosphate; DHF, dihydrofolate; THF, tetrahydrofolic acid; ATP, adenosine triphosphate; GTP, guanosine triphosphate

Enzymes: PPAT, phosphoribosyl pyrophosphate amidotransferase; GART, phosphoribosylglycinamide formyltransferase; FGAMS, formylglycinamidine ribonucleotide synthase; PAICS, phosphoribosylaminoimidazole–succinocarboxamide synthetase; ADSL, adenylosuccinate lyase; ATIC, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; APRT, adenine phosphoribosyltransferase; IMPDH, inosine monophosphate dehydrogenase; GMPS, GMP synthetase; 5'-NT, 5'-nucleotidase; PNP, purine nucleoside phosphorylase; ADSS, adenylosuccinate

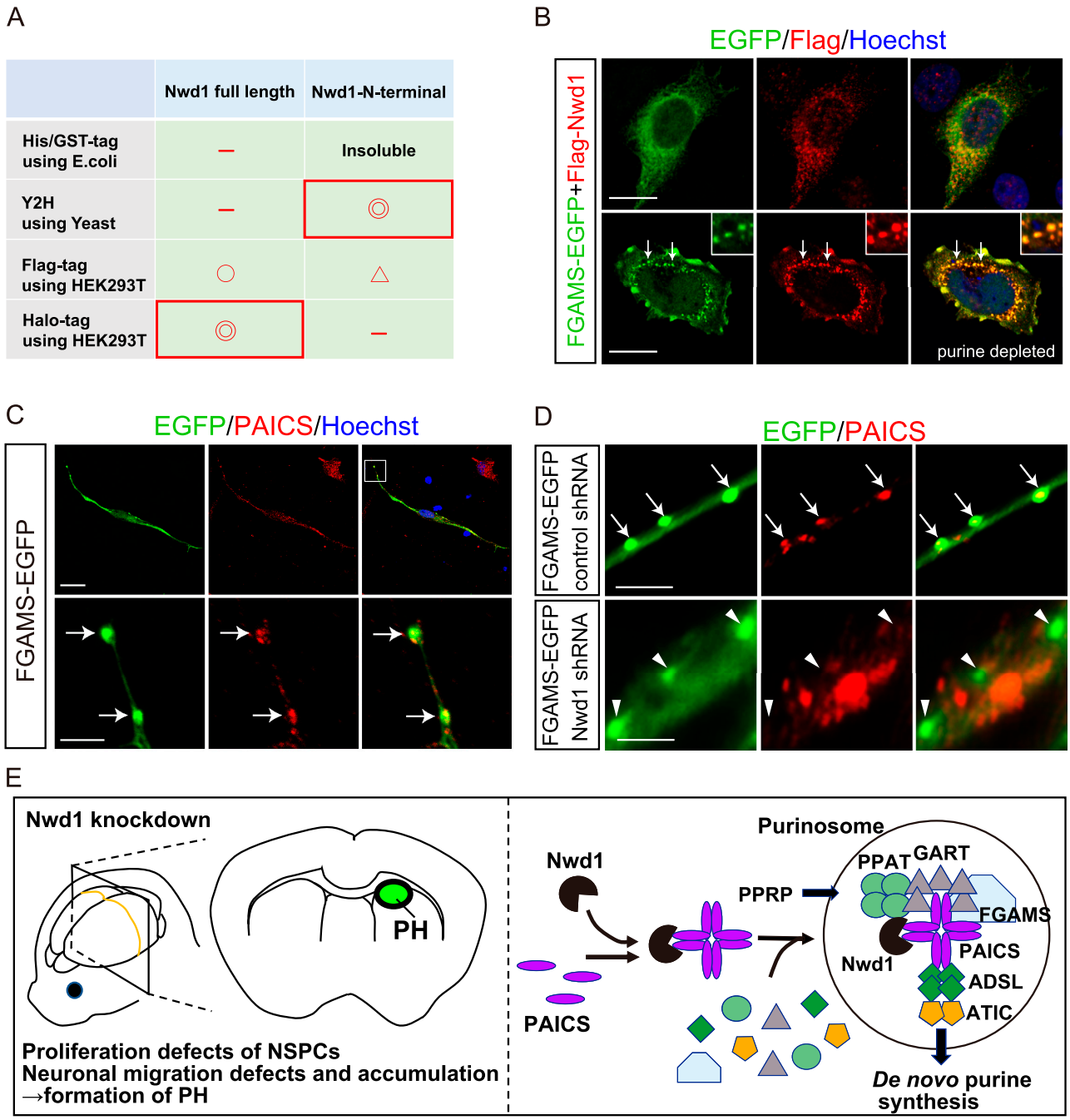


Fig. 5. The role of Nwd1 in purinosome formation in NSPCs [80]. (**A**) Methods for screening Nwd1-binding proteins [80, 81]; (**B**) Flag-Nwd1 and FGAMS-EGFP co-localized in purinosomes in HeLa cells under purine-depleted conditions; (**C**) FGAMS-EGFP expression co-localized with endogenous PAICS as purinosome in primary cultured NPSCs; (**D**) Decreased formation of $FGAMS^{+}$ $PAICS^{+}$ purinosomes was seen in primary cultured NSPCs expressing Nwd1 shRNA. FGAMS-EGFP was introduced into NSPCs to monitor the purinosome formation; (**E**, left panel) Schematic model of brain abnormalities caused by Nwd1 knockdown. Inhibition of Nwd1 expression caused proliferation defects of NSPCs, premature differentiation into immature neurons, and delayed neuronal migration, resulting in the formation of PH; (**E**, right panel) Hypothetical molecular model of purinosome assembly. The Nwd1–PAICS complex systematically tethers other enzymes, such as FGAMS, PPAT, GART, ADSL, and ATIC, forming a functional purinosome [Bars = 15 µm in (**B**); 20 µm in (**C**, upper); and 4 µm in (**C**, lower), and (**D**)].

synthetase isozyme 2; ADA, adenosine deaminase; XO, xanthine oxidase; DHFR, dihydrofolate reductase; SHMT, Serine hydroxymethyltransferase, cytosolic; MTHFD1, methylenetetrahydrofolate dehydrogenase; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase

Chemical inhibitor: LTX, lometrexol; AG 2037, Pelitrexol; MRT, MRT00252040; MMF, mycophenolate mofetil; MZR, mizoribine; Forodesine, forodesine

hydrochloride; DAP, 2,6-diaminopurine; MTX, methotrexate; SHIN1, SHMAT inhibitor 1; AZ, azathioprine; 6-MP, 6-mercaptopurine

VIII. Conflicts of Interest

The authors have no conflicts of interest to declare.

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