Reinke crystals are immunoreactive for purine-synthesizing metabolic enzymes

John Woulfe MD PhD: Department of Pathology and Laboratory Medicine, University of Ottawa and Ottawa Hospital Research Institute, Ottawa, Ontario, Canada

Trevor Flood MD: Department of Pathology and Laboratory Medicine, University of Ottawa, Ottawa, Ontario, Canada

Sharlene Faulkes PhD: Department of Pathology and Laboratory Medicine, University of Ottawa, Ottawa, Ontario, Canada

David G. Munoz MD: Department of Laboratory Medicine, St. Michael's Hospital, Unity Health & Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

Pages: 20
Tables: 0
Figures: 1
Running head: Metabolic enzymes in Reinke crystals

Corresponding author:
John Woulfe
Dept. of Pathology, The Ottawa Hospital, Civic Campus
1053 Carling Avenue, Ottawa, Ontario, Canada
K1Y 4E9
Phone: 613-798-555 ext. 13345
Fax: 613-761-4199
E-mail: jwoulfe@toh.ca
ORCID ID: 0-0002-0007382-6343

Conflicts of interest: The authors have no relevant financial or non-financial conflicts of interest to disclose.

Funding: No funding was received for conducting this study.

Key words: Reinke crystals, Leydig cells, nucleotides, inosine monophosphate dehydrogenase, phosphoribosyl pyrophosphate synthetase, GTP

Abstract

Reinke crystals are a defining histological feature of human adult Leydig cells, the testosterone producing cells of the testis. These morphologically-striking structures are present in the cytoplasm and the nucleus and display quantitative alterations in a variety of physiological and pathological contexts. The functional significance and protein composition of Reinke crystals have remained elusive for over a century. Here, we demonstrate that Reinke crystals are intensely immunoreactive for inosine monophosphate dehydrogenase (IMPDH), and phosphoribosyl pyrophosphate synthetase (PRPS), two key enzymes in the *de novo* synthesis of purine nucleotides. IMPDH and PRPS are two of several metabolic enzymes that is capable of forming mesoscale filamentous aggregates as a mechanism to regulate enzyme activity. IMPDH is also able to form crystals *in cellulo*. Our observations link Reinke crystal formation to purine nucleotide metabolism in Leydig cells. We discuss how this novel finding may relate to the unique dependence of Leydig cells on GTP for testosterone synthesis. The results of this preliminary study may have important implications for understanding the mechanisms underlying disorders of male reproductive health as well as offering a novel histological biomarker for Leydig cell neoplasms. Moreover, we have previously demonstrated similar IMPDH/PRPS structures in the human brain, suggesting that this phenomenon may have broad biological relevance across tissue types.

Introduction

Leydig cells, the testosterone-producing cells of the testis, are essential for male reproductive health [1]. Reinke crystals, first described by Frederich B. Reinke in 1896 [2], are a defining cytological feature of both native and neoplastic Leydig cells (for a review, see [3]). The presence of Reinke crystals in neoplastic Leydig cells is of diagnostic utility [4]. They are well-circumscribed, hyaline eosinophilic structures using conventional hematoxylin and eosin staining and are also demonstrable in Masson's trichrome, Giemsa, and Gram preparations [4-7]. Reinke crystals are 2-3 μm in size on average but can measure up to 20 μm [8]. Ultrastructurally, their morphology is consistent with true crystals, showing "long-range order" in which 5 nm parallel filaments are arranged in a regular array throughout the entire structure. The protein composition and functional significance of Reinke crystals have remained enigmatic. Their abundance is dependent on Leydig cell developmental stage[9] and is altered in a variety of testicular disorders (see [3]).

To date, candidate proteins that have been implicated as Reinke crystal constituents include nestin [10] and a 3β hydroxysteroid/calretinin complex [7, 11]. However, definitive confirmation of is lacking. The search for protein candidates has focused on those whose expression is confined to or enriched in Leydig cells. The rationale for this seems to be based on the reasoning that protein crystals like those described by Reinke are exclusive to Leydig cells. However, native crystals of unknown protein composition have been described in other cell types and tissues [12]. In this context, it is equally plausible that some property shared by distinct cell types leads to crystallization of a protein common to many cells.

Inosine monophosphate dehydrogenase (IMPDH) catalyzes the first committed step in the *de novo* synthesis of guanine-based purine nucleotides, most notably guanosine triphosphate

(GTP). GTP is an essential building block of RNA and DNA, is used as a signalling molecule in several molecular pathways, and is incorporated into co-enzymes. There are two isoforms of IMPDH that share 83% amino acid identity and have differential expression patterns [13, 14]. IMPDH1 is expressed constitutively albeit at low levels in many tissues whereas IMPDH2 expression is also ubiquitous but is upregulated in proliferating tissues [15-18]. IMPDH is one of a growing list of metabolic enzymes capable of regulated assembly to form long filaments as a mechanism to adjust enzyme availability and activity [19-21]. Individual enzyme filaments coalesce to form microscopically visible cytoplasmic or intranuclear filament bundles called "cytoophidia" (Gk. For "cellular snakes") [21]. Importantly, IMPDH can also form large intracellular crystals [22]. This spectrum suggests a temporally sequential series of assembly states in which crystallization represents the ultimate stage.

Although all cells require purine nucleotides such as GTP, some are particularly GTP-dependent. *In vivo*, IMPDH filament assemblies serve as a marker for such cells [19, 20, 23]. We have described IMPDH-immunoreactive rod-shaped or rhomboid paracrystalline structures within the cytoplasm and nucleus of dopamine (DA) producing neurons of the human substantia nigra (SN) [24]. We showed that these neuronal IMPDH filaments showed co-staining for phosphoribosyl pyrophosphate synthetase (PRPS) [25, 26]. PRPS is another filament-forming enzyme in the purine biosynthesis pathway which catalyzes the conversion of ribose-5-phospahte to phosphoribosyl pyrophosphate in one of the first steps in *de novo* purine nucleotide synthesis [25, 27-29].

Consistent with the presence of IMPDH/PRPS assemblies, several factors would support the prediction that SN DA neurons have a particularly high demand for GTP. For example, GTP is required as a co-factor for the microtubule polymerization and remodelling necessary to

support the elaboration and maintenance of the massively divergent axonal arborization of nigrostriatal neurons [30]. Moreover, it is a substrate for the myriad ras-associated binding (Rab) GTPases charged with the task of transporting vesicles and organelles along this expansive microtubule network [31]. GTP is also a requisite precursor for tetrahydrobiopterin (BH_4), an essential cofactor for tyrosine hydroxylase (TH), the rate-limiting enzyme in DA production [32]. By analogy, Leydig cells are also uniquely GTP-dependent [33]. They produce testosterone in response to LH-mediated cAMP generation via a G-protein-coupled receptor [1]. Whereas most endocrine cells use GTP as a signalling molecule to some extent, they can exploit alternate pathways, whereas testosterone synthesis in adult Leydig cells is exclusively dependent on GTP as a substrate for luteinizing hormone (LH)-mediated receptor signalling [33]. Thus, both cell types are therefore not only metabolically active but also functionally constrained by the availability of GTP, linking purine nucleotide metabolism directly to their core physiological roles and making them uniquely vulnerable to disruptions in purine homeostasis.

Recognizing: 1) the shared dependence of SN DA neurons and Leydig cells on GTP, 2) the morphological similarity of Reinke crystals to our SN DA neuron nuclear IMPDH crystals [34] and to the *in cellulo* IMPDH crystals described previously [22], and 3) the fact that Reinke crystals, like our SN DA neuron IMPDH/PRPS crystals can be localized to the nucleus [5], we were compelled to investigate whether Reinke crystals are also immunoreactive for the purine nucleotide-synthesizing enzymes IMPDH and PRPS.

Materials and Methods

The study was conducted in accordance with the ethical standards of The Ottawa Hospital. Tissue blocks of formalin-fixed, paraffin-embedded tissue from four cases of testicular

Leydig cell tumours with adjacent non-neoplastic testis were obtained from the tissue archive in the Division of Anatomical Pathology of the Ottawa Hospital. These were resected from four male participants aged 28, 29, 70, and 71. Diagnoses in all cases were well-circumscribed Leydig cell tumours confined to the testicular parenchyma. The tumours measured from 1.3 to 1.6 cm. A representative block from each case was sectioned at a thickness of 5 μm, and sections were mounted onto coated slides. Immunohistochemical staining was performed on tissue sections using the Leica Bond™ system. Sections were deparaffinized and pre-treated using heat mediated antigen retrieval with an EDTA buffer (pH 9) for 20 minutes. The sections were then incubated in rabbit polyclonal anti-IMPDH1 (1:400; Proteintech #22092-1 -AP, Lot #00018215; Rosemont IL USA) or rabbit polyclonal PRPS1 (1:200; ProteinTech #15549-1-AP) for 30 minutes at room temperature and detected using an HRP conjugated compact polymer system. Slides were then stained using DAB as the chromogen, counterstained with hematoxylin, mounted and cover-slipped. As an experimental control for antibody specificity, the IMPDH primary antibody was pre-absorbed using a 10 fold molar excess (~14 ug) of the immunizing peptide (ProteinTech #Ag17473), and incubated overnight at 4 degrees with gentle agitation which reduced staining by 90%. For method specificity, omission of the primary antibodies resulted in a complete absence of staining. Microscopic imaging was performed using Nikon Eclipse 80i light microscope. Photomicrographs were acquired using a Nikon Digital Sight Fi-1 camera and processed using NIS Elements imaging software version 4.60.

Results

In all 4 cases, cytoplasmic Reinke crystals were visualized in hematoxylin- and eosin (H and E)-stained sections in both tumour cells and native Leydig cells (Figure 1). Their frequency

varied among cases. Reinke crystals were most apparent in the cytoplasm where they appeared as well-circumscribed eosinophilic structures measuring from approximately 2 to 30 μm (Figure 1A). They were often rod-shaped or rectangular although various more irregular morphologies were common. Larger Reinke crystals, particularly the rod-shaped structures, appeared to "stretch" the plasma membrane of the host cell. In this limited series, we did not detect consistent differences in the size or morphology of cytoplasmic Reinke crystals between neoplastic and non-neoplastic Leydig cells in H and E-stained sections.

Reinke crystals were also visualized within the nuclei of neoplastic and non-neoplastic Leydig cells in H and E-stained sections (Figures 1A and B; arrowheads). The majority of these consisted of an amorphous eosinophilic area devoid of hematoxylin staining. In rare cases, nuclear crystals with a more solid rod-shaped morphology could be identified (Figure 1B). Some of these were longer than the nuclear diameter and distorted the nuclear membrane.

Across all four cases, both cytoplasmic and nuclear Reinke crystals displayed intense immunoreactivity for IMPDH and PRPS, concordant with the crystalline profiles seen on H&E (Figure 1B and C). The intensity and distribution of IMPDH and PRPS staining with respect to Reinke crystals was identical. In both Leydig cell tumours (Figure 1A-E) and native Leydig cell nests (Figure 1F), Reinke crystals were far more evident and abundant in IMPDH and PRPS-immunostained sections relative to those stained using H and E (compare Figure 1A with C and D). For example, whereas less than half of the Leydig cell population appeared to have nuclear Reinke crystals in the latter, IMPDH or PRPS immunostaining revealed nuclear Reinke crystals in approximately 90% of the neoplastic Leydig cell population (Figure 1E). Moreover, immunostaining revealed features that were not appreciated on H and E staining. For example,

whereas cytoplasmic crystals were intensely stained and showed well-defined edges, many nuclear crystals showed “blush” staining and ill-defined margins (Figure 1E, arrowheads).

Discussion

Despite having been described over a century ago [2], the protein composition of Reinke crystals has remained a mystery. Here, we demonstrate intense IMPDH and PRPS immunoreactivity within Reinke crystals. Nass and colleagues demonstrated spontaneous IMPDH crystal formation in Sf9 insect cells transfected with *T. brucei* IMPDH [22]. Our study indicates that human IMPDH may also be capable of crystal formation in human Leydig cells. The presence of IMPDH crystals in both neurons [24] and Leydig cells indicates that intracellular IMPDH crystal formation may have broad biological relevance across tissue types.

Whether PRPS is capable of spontaneous crystallization *in cellulo* has not been investigated. However, consistent with our previous studies [25, 26] as well as *in vitro* studies (Hvorecny, personal communication) showing co-localization of IMPDH and PRPS in cytoophidia, we show here that Reinke crystals stain intensely for both of these purine nucleotide synthesizing enzymes. The co-assembly of distinct metabolic enzymes to form heterotypic assemblies is an emerging theme in the field of enzyme filamentation [27, 35]. Interestingly, co-assembling enzymes tend to be those located at critical "branch-points" in the purine biosynthetic pathway, including PRPS and IMPDH, suggesting that co-assembly may have an important regulatory role [27].

We used polyclonal antibodies generated against IMPDH1 and PRPS1. Our study does not distinguish the various IMPDH and PRPS isoforms. The amino acid sequences of IMPDH 1 and 2 are highly homologous. The antibody used in this study was generated using a synthetic

peptide immunogen corresponding to amino acids 170–235 of IMPDH1. This sequence shares 77% homology with amino acids 121–186 of IMPDH2. Consequently, it is not possible to determine whether the structures we have described here are comprised of IMPDH 1, 2, or a combination of these isoforms. Keppeke and co-workers demonstrated co-localization of both isoforms in cytoophidia, including intranuclear structures, in mammalian cells [19]. Similarly, there are 3 isoforms of PRPS (PRPS1, 2, and 3) in humans. The antibody used in the present study was generated against amino acids 1-318 of PRPS1 which share 98.7% and 98.1% sequence identity with PRPS2 and PRPS3, respectively.

Like the intranuclear structures in SN DA neurons, the results of our study link Reinke crystals to purine nucleotide, and specifically to guanine-based (GTP) metabolism. Although the presence of PRPS, which catalyzes one of the first steps in the purine biosynthesis pathway, possibly implicates adenine-based purine nucleotide metabolism as well, we have been unable to demonstrate the presence, in cytoophidia or crystals, of more downstream enzymes in the AMP/ADP/ATP *de novo* synthesis pathway. Leydig cells are uniquely reliant on GTP for testosterone synthesis. They produce testosterone in response to luteinizing hormone (LH) binding to a G-protein-coupled receptor, with subsequent GTP-mediated activation of Gsα and the generation of cAMP. The latter stimulates cholesterol translocation to mitochondria where it is metabolized to pregnenolone which in turn is converted to testosterone by enzymes in the endoplasmic reticulum [1]. Whereas all endocrine cells utilize GTP signaling to some extent, adult Leydig cells lack redundancy in this respect and are exclusively dependent on the LH-GTP-cAMP signaling axis [33]. In contrast, fetal Leydig cells as well as testosterone-producing adrenal cells, both of which lack Reinke crystals, can produce testosterone through non-GTP-dependent mechanisms [33, 36].

Understanding the contribution of IMPDH and PRPS to the crystal structure, as well as their functional status with respect to enzyme activity in the crystalline context, are key prerequisites to elucidating the significance of Reinke crystal formation. With respect to structure, further studies, such as immunostaining studies at the electron microscopic level, are required to confirm that these two enzymes represent the core component of the filaments in the crystal lattice. In support of this possibility, in contrast to other proteins that have been nominated as Reinke crystal constituents, both IMPDH and PRPS assemble into macrostructures, including, for IMPDH, crystals with a light microscopic and ultrastructural morphology similar to that described for Reinke crystals [22, 37]. In terms of function, soluble IMPDH functions as a tetramer [38]. The enzyme converts inosine monophosphate (IMP) to xanthosine monophosphate (XMP), which is then converted by other enzymes sequentially to GMP, GDP, and GTP. In response to allosteric regulators, including ATP and GTP, IMPDH tetramers assemble into octamers. Adenine and guanine nucleotides differentially modulate the three-dimensional conformation of the octamer by binding to allosteric sites in what has been called a "conformational switch" [39]. Binding of adenine nucleotides results in an "extended", active conformation, in which the IMPDH catalytic sites are exposed to substrates, whereas binding of GTP/GDP results in a more enzymatically inactive, "compressed" conformation [40]. On further exposure to ATP or GTP individual octamers stack to form filaments [41, 42]. Individual filaments consist of a mosaic of octamers in both the extended and compressed states [40, 41]. Filament formation sustains GTP synthesis by resisting feedback inhibition by GTP [40]. In response to further stimuli that alter IMPDH catalytic flux [19, 20, 43, 44] IMPDH filaments coalesce into the micron-scale structures composed of bundled filaments ("cytoophidia") [19, 20, 43-47]. The presence of cytoophidia *in vivo* is a morphological marker for cells with high nucleotide demand.

Studies suggest that these filament bundles are enzymatically active, serving to sustain nucleotide levels [19, 48]. However, cytoophidia formation also prolongs the half-life of IMPDH, suggesting that they may also have a storage function [49]. Whether IMPDH crystals are enzymatically active is unknown. Whereas individual IMPDH filaments and those in bundles (cytoophidia) are composed of both extended (enzymatically active) and compact (inactive) [39] octamers, the regular, ordered nature of the filaments in the crystal suggests that the octamers are all uniformly in either the extended or compact conformation. Based on their identification of a novel GMP co-ordination site in *T. brucei* IMPDH crystals, Nass and colleagues provided evidence that IMPDH octamers were in the compact (inactive) conformation [22]. This suggests that IMPDH crystals may be enzymatically inert. PRPS cytoophidia have also been described and, like IMPDH, are thought to promote allosteric stimulation of catalytic activity [28] . However, spontaneous PRPS crystallization has not hitherto been demonstrated in cells. Our studies suggest that both of these key purine synthesizing enzymes co-localize in Reinke crystals. It is tempting to speculate that this reflects a transition to long range order of the IMPDH/PRPS double immunoreactive filaments that we [25, 26] and others (Hvorecny, personal communication) have demonstrated *in vivo* and *in vitro*, respectively. Future cryo-electron microscopic studies of Reinke crystals might confirm the presence of IMPDH/PRPS oligomers and reveal the nature of their interaction in the crystalline context.

The prediction that IMPDH/PRPS crystals are enzymatically inert has implications for understanding their functional significance. Intracellular protein crystals have been described *in vivo* in a variety of cell types. They can be classified as "accidental" crystals, sometimes caused by mutations of the constituent protein(s) and "native" crystals which have a physiological role. Whereas accidental crystal formation imposes cytotoxic consequences culminating in diseases

like cataracts and sickle cell disease [12, 50, 51], native crystals are purported to serve as protein storage depots. Examples include insulin crystals in the exocytotic vesicles of pancreatic beta cells and major basic protein 1 in eosinophils [12, 52, 53]. By analogy, cytoplasmic Reinke crystals in Leydig cells might provide a readily accessible storage compartment for IMPDH and PRPS to be deployed during periods of increased nucleotide demand. Consistent with this, in conditions of higher LH levels and Leydig cell hyperplasia, including 17-βHSD deficiency and androgen insensitivity syndrome, Reinke crystals are reduced or not present [3, 54, 55]. We propose that this is a result of translocation of these key enzymes from the crystal to satisfy the requirements imposed by upregulated LH signaling. This hypothesis could be extended to nuclear Reinke crystals [5, 8]. In SN DA neurons, IMPDH/PRPS crystals show consistent localization adjacent to the nucleolus [24]. The nucleolus consumes vast quantities of GTP for the synthesis of ribosomal RNA [56, 57]. Juxtanucleolar crystals may provide a proximate “hyperlocal” reservoir of IMPDH/PRPS to be deployed for the synthesis of GTP. Alternatively, or in addition, IMPDH has been described in the cell nucleus where it has been ascribed a non-enzymatic "moonlighting" role [58]. Specifically, nuclear IMPDH binds to DNA and functions as a transcription factor, regulating E2F-dependent gene expression in a cell-cycle dependent manner. Similarly, a non-enzymatic moonlighting role for PRPS in nuclear histone deposition has been demonstrated [59]. In this context, Leydig cells might exploit the capacity of IMPDH and PRPS to form crystals as a mechanism to dynamically regulate their nucleoplasmic availabilities to perform these moonlighting functions. Although intranuclear PRPS cytoophidia have been described [25-27] , whether PRPS has a similar non-enzymatic nuclear function is unknown. Finally, it is also conceivable that nuclear Reinke crystals simply represent an "overflow" storage site for excess cytoplasmic IMPDH/PRPS.

Nuclear Reinke crystals appeared to be less intensely stained and were more morphologically amorphous than the "rigid"-appearing cytoplasmic crystals. This may relate to observations suggesting that nuclear Reinke crystals may be at an earlier, precursor stage of formation, corresponding to paracrystalline structures [5, 60].

The observation that children with Lesch-Nyhan syndrome, the prototypical disorder of purine metabolism, display impaired testicular development and testosterone deficiency [61] reinforces the link between Leydig cell function and purine metabolism in a pathophysiological context with potential implications for understanding the pathogenesis of male reproductive disorders. There may also be implications for cancer diagnosis and treatment. Leydig cell tumours are a rare form of testicular cancer. Most of these are benign although malignant variants can occur with metastatic potential. The identification of Reinke crystals in cytological preparations or tissue sections is useful in diagnosing these tumours [4]. However, many tumours have very few crystals. In addition, Reinke crystals are sensitive to formalin fixation, contributing to false negative interpretation in H and E-stained sections [7]. Immunohistochemistry for the detection of IMPDH or PRPS provides a novel, more sensitive histological marker for Reinke crystal identification in the diagnostic setting. With respect to therapeutic implications, several studies suggest that, under steady-state conditions, IMPDH filamentation is a hallmark of cells in which guanine nucleotide pools are limiting [19, 23, 62]. This may represent a metabolic vulnerability that could be leveraged by IMPDH inhibitors to further deplete GTP, culminating in DNA replication stress and cell death. An important corollary of this is that the immunohistochemical detection of Reinke crystals in Leydig cell tumours may provide a histological biomarker predictive of a favourable therapeutic response to IMPDH inhibitors. Currently approved IMPDH inhibitors like mycophenolic acid as well as newer generation

agents are receiving renewed attention as anti-neoplastic agents [63]. Future preclinical studies and clinical trials should assess the susceptibility of Reinke-crystal bearing Leydig cell malignancies to IMPDH inhibition.

Further studies are necessary to consolidate the results of this preliminary investigation. Our observations are based on single polyclonal anti-IMPDH1 and anti-PRPS1 reagents. Additional validation using other antibodies targeting differential isoform-specific epitopes is warranted, (with the caveat that antibody binding may in many cases be precluded due to epitope hiding in the very compact crystal structure). The present series is limited to cases from a single institution. Generalizability across age groups, institutions, and testicular disorders remains to be established. Furthermore, we did not assess specificity against other testicular neoplasms or non-Leydig cell components. As indicated above, it will be important to conduct immunogold electron microscopy to localize IMPDH and PRPS at nanometer resolution within the paracrystalline lattice of Reinke crystals. This could be supplemented by laser-capture microdissection of crystals followed by LC-MS/MS to identify IMPDH and PRPS peptides, and pinpoint their isotype composition, enriched in crystal material.

Conclusions

Reinke crystals, a defining cytological feature of Leydig cells that has remained enigmatic for over a century, are immunoreactive for IMPDH and PRPS, linking these structures to purine nucleotide metabolism and possibly a role in regulating GTP availability. In addition to solving a long-standing mystery, this finding has important implications for understanding the role of purine nucleotide metabolism in testicular function and disease, including cancer. Moreover, our previous demonstration of similar structures in SN DA neurons suggests that

IMPDH/PRPS crystal formation may have broader biological relevance across diverse tissue types.

Acknowledgements

The authors would like to acknowledge the excellent assistance of Dr. Sharlene Faulkes and the Louise Pelletier Histology Core Facility (RRID: SCR_021737), Department of Pathology and Laboratory Medicine, University of Ottawa.

Author Contributions

J.W. designed the project, performed experiments, analyzed the data, and wrote the manuscript; T.F. provided tissue for the project, provided guidance regarding significance with respect to Leydig cell function and edited the manuscript; S.F. performed the immunohistochemical experiments and edited the manuscript; D.G.M. edited and provided critical input regarding organization of the manuscript. J.W. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Figure Legend:

Figure 1: Reinke crystals are immunoreactive for IMPDH and PRPS. A-D: Reinke crystals in a Leydig cell tumour. A) Cytoplasmic (arrows) and nuclear (arrowheads) Reinke crystals are visible in an H and E stained section. B) A rod-shaped nuclear crystal extends to a length greater than the nuclear diameter, distorting and stretching the nuclear membrane (arrowheads). C, D) Immunostaining for IMPDH (C) and PRPS (D) reveals more abundant Reinke crystals

morphologically identical to those visualized in H and E-stained sections. E) IMPDH immunohistochemistry is more sensitive for the demonstration of nuclear Reinke crystals. Many nuclear crystals (eg. arrowheads) stain with reduced intensity relative to cytoplasmic crystals. F) Both cytoplasmic and nuclear crystals are visualized in non-neoplastic Leydig cells using IMPDH immunohistochemistry. Scale bars=50 μm in A, C, and D; 25 μm in B, E, and F.

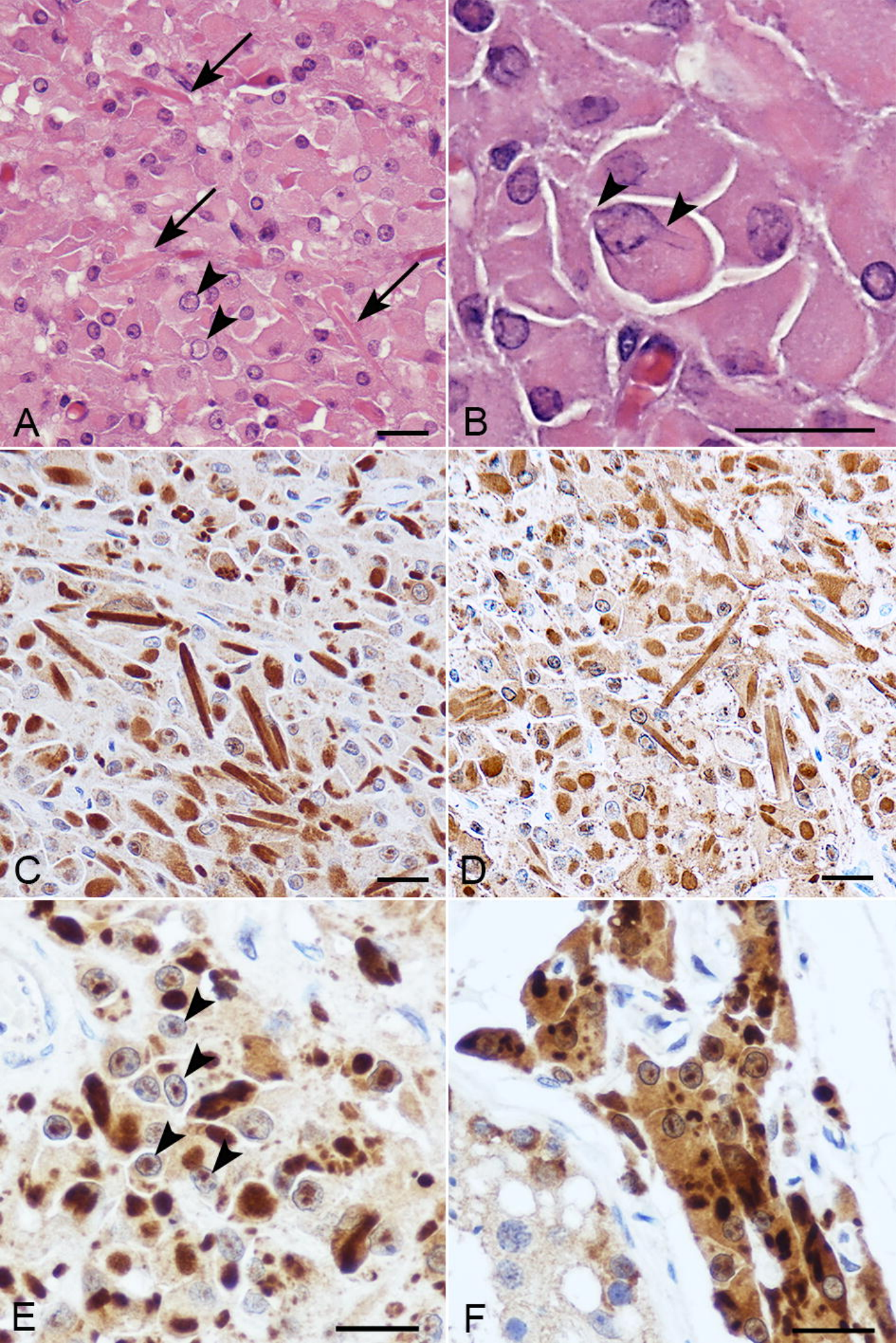
References

[1] Zirkin BR, Papadopoulos V: Leydig cells: formation, function, and regulation. Biol Reprod 2018, 99:101-11.
[2] F R: Beitrage zur histologie des menschen. Archiv fur mikroskopische Anatomie 1896, 47:34-44.
[3] Planinic A, Maric T, Bojanac AK, Jezek D: Reinke crystals: Hallmarks of adult Leydig cells in humans. Andrology 2022, 10:1107-20.
[4] Handa U, Sood T, Punia RS: Testicular Leydig cell tumor diagnosed on fine needle aspiration. Diagnostic cytopathology 2010, 38:682-4.
[5] Gupta SK, Francis IM, Sheikh ZA, al-Rubah NA, Das DK: Intranuclear Reinke's crystals in a testicular Leydig cell tumor diagnosed by aspiration cytology. A case report. Acta Cytol 1994, 38:252-6.
[6] Jain M, Aiyer HM, Bajaj P, Dhar S: Intracytoplasmic and intranuclear Reinke's crystals in a testicular Leydig-cell tumor diagnosed by fine-needle aspiration cytology: a case report with review of the literature. Diagnostic cytopathology 2001, 25:162-4.
[7] Mesa H, Gilles S, Smith S, Dachel S, Larson W, Manivel JC: The mystery of the vanishing Reinke crystals. Hum Pathol 2015, 46:600-6.
[8] Kozina V, Geist D, Kubinova L, Bilic E, Karnthaler HP, Waitz T, Janacek J, Chernyavskiy O, Krhen I, Jezek D: Visualization of Reinke's crystals in normal and cryptorchid testis. Histochemistry and cell biology 2011, 135:215-28.
[9] Prince FP: Ultrastructural evidence of mature Leydig cells and Leydig cell regression in the neonatal human testis. Anat Rec 1990, 228:405-17.
[10] Lobo MV, Arenas MI, Alonso FJ, Gomez G, Bazan E, Paino CL, Fernandez E, Fraile B, Paniagua R, Moyano A, Caso E: Nestin, a neuroectodermal stem cell marker molecule, is expressed in Leydig cells of the human testis and in some specific cell types from human testicular tumours. Cell and tissue research 2004, 316:369-76.
[11] Mesa H, Gilles S, Datta MW, Murugan P, Larson W, Dachel S, Manivel C: Immunophenotypic differences between neoplastic and non-neoplastic androgen-producing cells containing and lacking Reinke crystals. Virchows Arch 2016, 469:679-86.
[12] Schonherr R, Rudolph JM, Redecke L: Protein crystallization in living cells. Biol Chem 2018, 399:751-72.
[13] Collart FR, Huberman E: Cloning and sequence analysis of the human and Chinese hamster inosine-5'-monophosphate dehydrogenase cDNAs. The Journal of biological chemistry 1988, 263:15769-72.
[14] Natsumeda Y, Ohno S, Kawasaki H, Konno Y, Weber G, Suzuki K: Two distinct cDNAs for human IMP dehydrogenase. The Journal of biological chemistry 1990, 265:5292-5.
[15] Carr SF, Papp E, Wu JC, Natsumeda Y: Characterization of human type I and type II IMP dehydrogenases. The Journal of biological chemistry 1993, 268:27286-90.
[16] Hager PW, Collart FR, Huberman E, Mitchell BS: Recombinant human inosine monophosphate dehydrogenase type I and type II proteins. Purification and characterization of inhibitor binding. Biochemical pharmacology 1995, 49:1323-9.
[17] Jackson RC, Weber G, Morris HP: IMP dehydrogenase, an enzyme linked with proliferation and malignancy. Nature 1975, 256:331-3.
[18] Senda M, Natsumeda Y: Tissue-differential expression of two distinct genes for human IMP dehydrogenase (E.C.1.1.1.205). Life Sci 1994, 54:1917-26.

[19] Keppeke GD, Chang CC, Peng M, Chen LY, Lin WC, Pai LM, Andrade LEC, Sung LY, Liu JL: IMP/GTP balance modulates cytoophidium assembly and IMPDH activity. Cell Div 2018, 13:5.
[20] Chang CC, Lin WC, Pai LM, Lee HS, Wu SC, Ding ST, Liu JL, Sung LY: Cytoophidium assembly reflects upregulation of IMPDH activity. Journal of cell science 2015, 128:3550-5.
[21] Calise SJ, Chan EKL: Anti-rods/rings autoantibody and IMPDH filaments: an update after fifteen years of discovery. Autoimmun Rev 2020, 19:102643.
[22] Nass K, Redecke L, Perbandt M, Yefanov O, Klinge M, Koopmann R, Stellato F, Gabdulkhakov A, Schonherr R, Rehders D, Lahey-Rudolph JM, Aquila A, Barty A, Basu S, Doak RB, Duden R, Frank M, Fromme R, Kassemeyer S, Katona G, Kirian R, Liu H, Majoul I, Martin-Garcia JM, Messerschmidt M, Shoeman RL, Weierstall U, Westenhoff S, White TA, Williams GJ, Yoon CH, Zatsepin N, Fromme P, Duszenko M, Chapman HN, Betzel C: In cellulo crystallization of Trypanosoma brucei IMP dehydrogenase enables the identification of genuine co-factors. Nature communications 2020, 11:620.
[23] Valvezan AJ, Turner M, Belaid A, Lam HC, Miller SK, McNamara MC, Baglini C, Housden BE, Perrimon N, Kwiatkowski DJ, Asara JM, Henske EP, Manning BD: mTORC1 Couples Nucleotide Synthesis to Nucleotide Demand Resulting in a Targetable Metabolic Vulnerability. Cancer cell 2017, 32:624-38 e5.
[24] Woulfe J, Munoz DG, Gray DA, Jinnah HA, Ivanova A: Inosine monophosphate dehydrogenase intranuclear inclusions are markers of aging and neuronal stress in the human substantia nigra. Neurobiol Aging 2024, 134:43-56.
[25] Woulfe J, Munoz D: Roncoroni Re-Visited: The Neuronal Intranuclear Rodlet Comes of Age. The Journal of comparative neurology 2024, 532:e25662.
[26] Ivanova A, Munoz DG, Woulfe J: Depletion of nuclear cytoophidia in Alzheimer's disease. Free Neuropathol 2025, 6:8.
[27] Noree C, Begovich K, Samilo D, Broyer R, Monfort E, Wilhelm JE: A quantitative screen for metabolic enzyme structures reveals patterns of assembly across the yeast metabolic network. Molecular biology of the cell 2019, 30:2721-36.
[28] Hvorecny KL, Hargett K, Quispe JD, Kollman JM: Human PRPS1 filaments stabilize allosteric sites to regulate activity. Nat Struct Mol Biol 2023, 30:391-402.
[29] Hove-Jensen B, Andersen KR, Kilstrup M, Martinussen J, Switzer RL, Willemoes M: Phosphoribosyl Diphosphate (PRPP): Biosynthesis, Enzymology, Utilization, and Metabolic Significance. Microbiol Mol Biol Rev 2017, 81.
[30] Matsuda W, Furuta T, Nakamura KC, Hioki H, Fujiyama F, Arai R, Kaneko T: Single nigrostriatal dopaminergic neurons form widely spread and highly dense axonal arborizations in the neostriatum. The Journal of neuroscience : the official journal of the Society for Neuroscience 2009, 29:444-53.
[31] Kiral FR, Kohrs FE, Jin EJ, Hiesinger PR: Rab GTPases and Membrane Trafficking in Neurodegeneration. Current biology : CB 2018, 28:R471-R86.
[32] Thony B, Auerbach G, Blau N: Tetrahydrobiopterin biosynthesis, regeneration and functions. The Biochemical journal 2000, 347 Pt 1:1-16.
[33] Sararols P, Stevant I, Neirijnck Y, Rebourcet D, Darbey A, Curley MK, Kuhne F, Dermitzakis E, Smith LB, Nef S: Specific Transcriptomic Signatures and Dual Regulation of Steroidogenesis Between Fetal and Adult Mouse Leydig Cells. Front Cell Dev Biol 2021, 9:695546.

[34] Woulfe J, Munoz DG, Gray DA, Jinnah HA, Ivanova A: Inosine monophosphate dehydrogenase intranuclear inclusions are markers of aging and neuronal stress in the human substantia nigra. Neurobiol Aging 2023, 134:43-56.
[35] Liu JL: The Cytoophidium and Its Kind: Filamentation and Compartmentation of Metabolic Enzymes. Annu Rev Cell Dev Biol 2016, 32:349-72.
[36] Mori Sequeiros Garcia MM, Paz C, Castillo AF, Benzo Y, Belluno MA, Balcazar Martinez A, Maloberti PM, Cornejo Maciel F, Poderoso C: New insights into signal transduction pathways in adrenal steroidogenesis: role of mitochondrial fusion, lipid mediators, and MAPK phosphatases. Front Endocrinol (Lausanne) 2023, 14:1175677.
[37] Nagano T, Otsuki I: Reinvestigation of the fine structure of Reinke's crystal in the human testicular interstitial cell. The Journal of cell biology 1971, 51:148-61.
[38] Hedstrom L: IMP dehydrogenase: structure, mechanism, and inhibition. Chemical reviews 2009, 109:2903-28.
[39] Buey RM, Fernandez-Justel D, Marcos-Alcalde I, Winter G, Gomez-Puertas P, de Pereda JM, Luis Revuelta J: A nucleotide-controlled conformational switch modulates the activity of eukaryotic IMP dehydrogenases. Scientific reports 2017, 7:2648.
[40] Johnson MC, Kollman JM: Cryo-EM structures demonstrate human IMPDH2 filament assembly tunes allosteric regulation. eLife 2020, 9.
[41] Anthony SA, Burrell AL, Johnson MC, Duong-Ly KC, Kuo YM, Simonet JC, Michener P, Andrews A, Kollman JM, Peterson JR: Reconstituted IMPDH polymers accommodate both catalytically active and inactive conformations. Molecular biology of the cell 2017.
[42] Fernandez-Justel D, Nunez R, Martin-Benito J, Jimeno D, Gonzalez-Lopez A, Soriano EM, Revuelta JL, Buey RM: A Nucleotide-Dependent Conformational Switch Controls the Polymerization of Human IMP Dehydrogenases to Modulate their Catalytic Activity. Journal of molecular biology 2019, 431:956-69.
[43] Ji Y, Gu J, Makhov AM, Griffith JD, Mitchell BS: Regulation of the interaction of inosine monophosphate dehydrogenase with mycophenolic Acid by GTP. The Journal of biological chemistry 2006, 281:206-12.
[44] Keppeke GD, Calise SJ, Chan EK, Andrade LE: Assembly of IMPDH2-based, CTPS-based, and mixed rod/ring structures is dependent on cell type and conditions of induction. J Genet Genomics 2015, 42:287-99.
[45] Calise SJ, Carcamo WC, Krueger C, Yin JD, Purich DL, Chan EK: Glutamine deprivation initiates reversible assembly of mammalian rods and rings. Cellular and molecular life sciences : CMLS 2014, 71:2963-73.
[46] Labesse G, Alexandre T, Vaupre L, Salard-Arnaud I, Him JL, Raynal B, Bron P, Munier-Lehmann H: MgATP regulates allostery and fiber formation in IMPDHs. Structure 2013, 21:975-85.
[47] Thomas EC, Gunter JH, Webster JA, Schieber NL, Oorschot V, Parton RG, Whitehead JP: Different characteristics and nucleotide binding properties of inosine monophosphate dehydrogenase (IMPDH) isoforms. PloS one 2012, 7:e51096.
[48] Calise SJ, Abboud G, Kasahara H, Morel L, Chan EKL: Immune Response-Dependent Assembly of IMP Dehydrogenase Filaments. Front Immunol 2018, 9:2789.
[49] Chang CC, Peng M, Zhong J, Zhang Z, Keppeke GD, Sung LY, Liu JL: Molecular crowding facilitates bundling of IMPDH polymers and cytoophidium formation. Cellular and molecular life sciences : CMLS 2022, 79:420.

[50] Pande A, Pande J, Asherie N, Lomakin A, Ogun O, King J, Benedek GB: Crystal cataracts: human genetic cataract caused by protein crystallization. Proceedings of the National Academy of Sciences of the United States of America 2001, 98:6116-20.
[51] Lawrence C, Fabry ME, Nagel RL: The unique red cell heterogeneity of SC disease: crystal formation, dense reticulocytes, and unusual morphology. Blood 1991, 78:2104-12.
[52] Dodson G, Steiner D: The role of assembly in insulin's biosynthesis. Curr Opin Struct Biol 1998, 8:189-94.
[53] Giembycz MA, Lindsay MA: Pharmacology of the eosinophil. Pharmacol Rev 1999, 51:213-340.
[54] Soerensen RR, Johannsen TH, Skakkebaek NE, Rajpert-De Meyts E: Leydig cell clustering and Reinke crystal distribution in relation to hormonal function in adult patients with testicular dysgenesis syndrome (TDS) including cryptorchidism. Hormones (Athens) 2016, 15:518-26.
[55] Chemke J, Shani A, Lancet M, Czernobilsky B: Testicular feminisation syndrome: unusual gonadal histology in an elderly patient. J Med Genet 1977, 14:375-8.
[56] Kofuji S, Hirayama A, Eberhardt AO, Kawaguchi R, Sugiura Y, Sampetrean O, Ikeda Y, Warren M, Sakamoto N, Kitahara S, Yoshino H, Yamashita D, Sumita K, Wolfe K, Lange L, Ikeda S, Shimada H, Minami N, Malhotra A, Morioka S, Ban Y, Asano M, Flanary VL, Ramkissoon A, Chow LML, Kiyokawa J, Mashimo T, Lucey G, Mareninov S, Ozawa T, Onishi N, Okumura K, Terakawa J, Daikoku T, Wise-Draper T, Majd N, Kofuji K, Sasaki M, Mori M, Kanemura Y, Smith EP, Anastasiou D, Wakimoto H, Holland EC, Yong WH, Horbinski C, Nakano I, DeBerardinis RJ, Bachoo RM, Mischel PS, Yasui W, Suematsu M, Saya H, Soga T, Grummt I, Bierhoff H, Sasaki AT: IMP dehydrogenase-2 drives aberrant nucleolar activity and promotes tumorigenesis in glioblastoma. Nature cell biology 2019, 21:1003-14.
[57] Kofuji S, Sasaki AT: GTP metabolic reprogramming by IMPDH2: unlocking cancer cells' fuelling mechanism. J Biochem 2020, 168:319-28.
[58] Kozhevnikova EN, van der Knaap JA, Pindyurin AV, Ozgur Z, van Ijcken WF, Moshkin YM, Verrijzer CP: Metabolic enzyme IMPDH is also a transcription factor regulated by cellular state. Molecular cell 2012, 47:133-9.
[59] Srivastava S, Samaniego-Castruita D, Srivastava S, Khurana S, Rehman J, Shukla V, Ben-Sahra I, Foltz DR: Rate-limiting enzymes in nucleotide metabolism synchronize nucleotide biosynthesis and chromatin formation. Molecular cell 2025.
[60] Kerr JB: Ultrastructure of the seminiferous epithelium and intertubular tissue of the human testis. J Electron Microsc Tech 1991, 19:215-40.
[61] Watts RW, Harkness RA, Spellacy E, Taylor NF: Lesch-Nyhan syndrome: growth delay, testicular atrophy and a partial failure of the 11 beta-hydroxylation of steroids. J Inherit Metab Dis 1987, 10:210-23.
[62] Duong-Ly KC, Kuo YM, Johnson MC, Cote JM, Kollman JM, Soboloff J, Rall GF, Andrews AJ, Peterson JR: T cell activation triggers reversible inosine-5'-monophosphate dehydrogenase assembly. Journal of cell science 2018, 131.
[63] Ogawa-Iio A, Takeuchi K, Shigemi K, Genoveso MJ, Niitsu H, Koh I, Ota Y, Yamane K, Hinoi T, Osaka N, Oshima M, Ishikawa T, Mizuno T, Natsumeda M, Tateishi K, Hashizume R, Osuka S, Goyama S, Yasuda T, Senda T, Sasaki AT: IMPDH and GTP Metabolism in Cancer: Mechanisms, Regulation, and Translational Scope. Cancer science 2025.


A
B
C
D
E
F