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ADP-ribose triggers neuronal ferroptosis via metabolic orchestrating

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Running title: ADP-ribose triggers neuronal cell death by ferroptosis

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Abstract

The depletion of NAD is intimately linked to neurodegenerative diseases and aging. Excessive activation of NAD^+-consuming enzymes that hydrolyze NAD to ADP-ribose and nicotinamide is closely related to neurodegenerative diseases. However, supplementation of NAD or its precursors failed to show any benefits for most patients. The molecular mechanisms underlying this are largely undefined. Here we show that ADP-ribose, as an endogenous NAD metabolite, triggers neuronal cell death by glutathione-independent ferroptosis. The neuronal cells in both the hippocampus and cerebral cortex were severely reduced under the treatment of ADP-ribose in mice. Intracellular ADP-ribose, an endogenous inhibitor of NAD-cofactor enzymes, but not NAD-consuming enzymes, is an accelerator of ferroptosis by blocking the catabolism of lipid peroxidation. Furthermore, the extracellular ADP-ribose directly binds to membrane surface nucleoside transporter ENT1 to orchestrate purine and pyrimidine metabolism. ENT1-hypoxanthine axis and glutamine-dihydroorotate-quinone pathway are independent and intersecting, which is specifically mobilized by ADP-ribose to trigger ferroptosis. Precisely because of it, we find that the neuronal death by ADP-ribose can be rescued by XO and DHODH inhibitors. Moreover, endogenous ADP-ribose levels are increased on oxidative stress and PROTAC of PARP1 maintain neuronal survival under these circumstances. We provide evidence that ADP-ribose is a key metabolite that is used for disease diagnosis and drug target. Our analyses uncover new molecular links between NAD metabolism and ferroptosis in the regulation of neuronal death, thus suggesting new strategies for the prevention and treatment of neurodegenerative diseases.

Key words: NAD metabolism, ADP-ribose, ferroptosis, ENT1, neurodegenerative diseases

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Introduction

Nicotinamide adenine dinucleotide (NAD), whose intracellular level is maintained between 0.2 and 0.5 mM(*1*), plays several essential roles in metabolism. It acts as a coenzyme in redox reactions, a donor of ADP-ribose moieties in ADP-ribosylation reactions(*2*), a substrate of enzymes called sirtuins that use NAD^+ to remove acetyl groups from proteins(*3, 4*), a precursor of the second messenger molecule cyclic ADP-ribose(*5, 6*), and a substrate for bacterial DNA ligases as well (*7*). In addition to those metabolic functions, NAD^+ emerges as an adenine nucleotide released from cells, hence can have important extracellular roles (*8, 9*). So the enzymes that produce and consume NAD are critical in both pharmacology and clinical research in future treatments for many diseases, such as but not limited to post-traumatic stress, anxiety, depression, chronic traumatic encephalopathy (CTE), Alzheimer's, Parkinson's, neurodegenerative diseases, aging and abuses of drugs and alcohol, which may induce the depletion of the natural amount of NAD(*10, 11*). However, supplementation of NAD or its precursors failed to show any benefits for most patients(*12-16*) in reality, which suggests that the initiation and progression of these diseases have other mechanisms than the depletion of NAD itself.

Hydrolysis of NAD by NAD^+-consuming enzymes

Based on its major functions, intracellular NAD-dependent enzymes can be classified into two types: NAD as a coenzyme in regulating the glucose-oxygen metabolic balance that controls the cellular ATP energy production through the NAD^+/NADH redox state, and NAD^+ as a co-substrate in modulating the activities of the NAD^+-consuming enzymes that mediate the cellular signaling processes through the availability of cellular NAD^+. In mammals, the two main NAD^+-consuming enzyme families are the sirtuins and the PARPs. Both protein families use NAD^+ as a co-substrate to modify target proteins, releasing nicotinamide (NAM). Sirtuins(*3, 4, 17, 18*), first discovered as yeast-silencing and telomere-protective proteins, regulate a wide variety of mammalian proteins involved in multiple processes including mitochondrial metabolism, inflammation, meiosis, autophagy, circadian rhythms, and apoptosis. The classic sirtuin reaction is the removal of an acetyl group from lysines on target proteins. The first step is to release NAM from NAD^+, followed by the formation of a peptidyl ADP-ribose intermediate covalently attached to the acetyl group of the lysine. Subsequently, the peptide chain with the targeted

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lysine is then liberated to yield o-acetyl-ADP-ribose. Then ADP-ribose glycohydrolase (ADPRS, OARD1, MACROD1/2) catalyzes the deacylation of O-acetyl-ADP-ribose, yielding ADP-ribose (ADPR) plus acetate. The other major NAD^+-consuming enzymes are the PARPs(*2, 19-21*), including both poly-ADP-ribose polymerases (PARP1, 2, and 5) and mono-ADP-ribose transferases, which is referred to MARTs (PARP3, 4, 6, 10, and 14–16). PARPs cleave NAD^+ and transfer the ADP-ribose moiety to asparagine, aspartic acid, glutamic acid, arginine, lysine, and cysteine residues on target proteins, forming branched poly-ADP-ribose polymers and releasing NAM in the process. Beyond proteins, ADP-ribosylation can be occured on nucleic acids. Poly(ADP-ribose) synthesized after DNA damage only presents transiently and is rapidly degraded by poly(ADP-ribose) glycohydrolase (PARG) that degrades poly(ADP-ribose) by hydrolyzing the ribose-ribose bonds present in poly(ADP-ribose). PARG acts both as an endo- and exoglycosidase, releasing poly(ADP-ribose) of different lengths as well as ADP-ribose monomers. It is however unable to cleave the ester bond between the terminal ADP-ribose and ADP-ribosylated residues, leaving proteins that are mono-ADP-ribosylated. ADP-ribose glycohydrolase (ADPRS, ADPRH, OARD1, MACROD1/2) that preferentially hydrolyzes the different mono-ADP-ribosylated substrates and free poly(ADP-ribose) to yield the ADP-ribose monomers.

NAD itself is also degraded by a group of enzymes called NAD nucleosidases (NADases). CD38 and CD157 (BST1) are NADases that cleave NAD to generate ADP-ribose and NAM, while CD38 also hydrolyzes cyclic ADP-ribose (cADPR) to ADP-ribose. These enzymes have been implicated in energy metabolism, cell adhesion, and various aspects of the immune response and have been linked to human diseases such as Parkinson's, ovarian cancer, and leukemia. To a lesser extent, both enzymes also act as ADP-ribosyl cyclases that catalyze the hydrolysis of NAD to generate NAM and cADPR, a Ca^{2+}-mobilizing second messenger active in many cell types(*6, 22*). SARM1 is a newly discovered NAD cleavage enzyme in neurons and possibly other cell types. It is the first member of a new class of NAD^+-consuming enzymes and unique among NADases in that its activity is dependent on SARM1's Toll/interleukin-1 receptor (TIR) domain. In response to neuronal injury, the catalytic TIR domain of SARM1 initiates a cell destruction program by converting cytoplasmic NAD to ADP-ribose, cADPR, and NAM. Therefore, SARM1 is an attractive therapeutic target for the treatment of acute neuronal damage and possibly neurodegenerative diseases(*23-26*).

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In short, NAD is eventually metabolized into ADP-ribose through multiple pathways. Furthermore, ADP-ribose pyrophosphatase (ADPRM and NUDT5/9/14) can hydrolyze ADP-ribose to AMP and ribose 5'-phosphate (R5P). R5P and its derivatives serve as precursors to many biomolecules, including DNA, RNA, ATP, coenzyme A, flavin adenine dinucleotide (FAD) and histidine. However, until now, the physiological and pathological functions of ADP-ribose, as an important metabolite of NAD, are still unknown.

NAD Consume enzymes and neurological diseases

Sirtuins are implicated in various biological pathways related to stress response, mitochondrial dysfunction, oxidative stress, protein aggregation and inflammatory processes that are intertwined with age-related neurodegenerative diseases. To the best of our knowledge, only the roles of SIRT1 and SIRT2 in neurodegeneration were extensively investigated. The findings show that SIRT2 inhibition or deletion generally displays a protective role against neurodegenerative diseases(*3, 27-31*).

DNA damage is the prime activator of the enzyme PARP-1 whose overactivation has been proven to be associated with the pathogenesis of numerous central nervous system disorders, such as ischemia, neuroinflammation, and neurodegenerative diseases. Under oxidative stress conditions PARP-1 activity increases, leading to an accumulation of ADP-ribose polymers and NAD+ depletion, inducing energy crisis and eventually cell death(*2, 19, 20, 32*).

While CD38 is strongly expressed in brain cells including neurons, astrocytes as well as microglial cells, the role played by CD38 in neurodegeneration and neuroinflammation remains elusive. Yet, CD38 expression increases as a consequence of aging which is otherwise the primary risk associated with neurodegenerative diseases, and previous studies demonstrated that CD38 knockout mice were protected from neurodegenerative and neuroinflammatory insults(*33*).

Although CD157 is expressed primarily in lymphoid tissue and gut, recent studies support that CD157 meaningfully acts in the brain as neuroregulators. Social behaviors are impaired in CD157 knockout mice. Single-nucleotide polymorphisms of the CD157/BST1 genes are associated with multiple neurological and psychiatric conditions, including autism spectrum disorder, Parkinson's disease, and schizophrenia(*34, 35*).

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SARM1 is known to be one of the main drivers of damage mechanisms that induce the degeneration of axons, extensions of nerve cells (also known as neurons) that are responsible for the transmission of the electrical signals within the central nervous system (brain and spinal cord). Its harmful enzymatic activity might be targeted by a new class of small chemical compounds to aid the development of targeted therapies for neurodegenerative disorders such as multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and peripheral neuropathies(*24-26, 36*).

In summary, it is not difficult to find that excessive activation of NAD Consume enzymes that consume NAD to produce ADP-ribose and nicotinamide is closely related to neurodegenerative diseases.

Ferroptosis

Ferroptosis is a mechanism for non-apoptotic, iron-dependent, oxidative cell death characterized by glutathione consumption and lipid peroxides accumulation. Ferroptosis is crucially involved in multiple neurological diseases, such as neurodegeneration, stroke and neurotrauma. Ferroptosis is a new type of cell death discovered in recent years and is usually accompanied by a large amount of iron accumulation and lipid peroxidation during the cell death process; the occurrence of ferroptosis is iron-dependent. Ferroptosis-inducing factors can directly or indirectly affect glutathione peroxidase through different pathways, resulting in a decrease in antioxidant capacity and accumulation of lipid reactive oxygen species (ROS) in cells, ultimately leading to oxidative cell death. Glutamate excitotoxicity is one of the mechanisms involved in cellular death in neurodegenerative diseases such as Alzheimer's disease or motor neuron disease (MND), which increases oxidative stress through activation of the glutamate receptors and inhibition of Cystine/glutamate antiporter xCT (SLC7A11) to trigger neuronal ferroptosis.

Results

ADP-ribose is a neuronal death factor

Although glutamate is the major excitatory neurotransmitter in the brain, excess extracellular glutamate may lead to excitotoxicity. It is well-known that excessive extracellular glutamate could serve as a natural trigger for inducing neuronal cell ferroptosis in physiological contexts

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through impacting system X_c^- function. EMX-A14 is a mouse homologue of the Drosophila homeobox gene empty spiracles and its expression is restricted to the neurons in the developing and adult cerebral cortex and hippocampus. We obtained neuronal cells from the cerebral cortex of embryonic 16.5 days EMX-A14 transgenic mice, which was labeled with red fluorescent protein (RFP) (Fig. S1A). It is interesting that when the neurons were treated with 5 mM glutamate for 6 hours and then replaced by the fresh medium to continue culturing for 6 hours, the collected medium also can induce neuronal cell death (Fig. S1B and 1A). Therefore we suspect that neurons responded to excessive glutamate may release certain factors into the extracellular environment to trigger neurons and adjacent cells death. Furthermore, the collected medium heated at 95℃ for 5 minutes is added to the fresh medium, which still kept promoting cell death (Fig. S1B and 1A). Moreover, the molecular sieve screening reveals that the size of the death factor is below 1,000Da (Fig. S1B and 1A).

In order to identify this death factor, integrating non-targeted and targeted metabolomics screening, we finally anchored the candidate small molecule metabolite ADP-ribose. Meanwhile, we used the QTrap 6500 to construct a standard measuring curve (ADP-ribose from Sigma, A0752) and measured the ADP-ribose concentration. Finally, we found that the intracellular and extracellular ADP-ribose concentration was significantly up-regulated under the stimulation of high-dose glutamate (Fig. 1B). We treated neuronal cells with different concentrations of ADP-ribose, and found that 100μM ADP-ribose was enough to kill most neuronal cells (Fig. 1C and S1C). Despite AMP and R5P are degradation products of ADP-ribose, they had no effect on cell death (Fig. 1D and S1D).

What is even more surprising is that the pro-death effect of ADP-ribose is specific towards neuronal cells, moreover, neuronal cell culture supplement B27 could significantly inhibit the effectiveness of ADP-ribose (Fig. 1E and S1E). To evaluate the role of ADP-ribose *in vivo*, hippocampus microinjection and cerebral cortex microinjection were performed in mice (Fig. 1F). One day after the drug intervention, we performed statistical analysis on the neuronal cells in the hippocampus and cerebral cortex of the rat, and found that the neuronal cells in both the hippocampus and cerebral cortex were severely reduced under the treatment of ADP-ribose (Fig. 1, G and I, S1F and S2A). It is obvious to see from figure S2A that the neurons in the CA1-CA3

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area have basically disappeared but the neurons in the DG area still survived, which may be due to the relatively long distance away from the injection point to DG neurons.

DNA damage is the prime activator of the enzyme poly(ADP-ribose)polymerase1 (PARP-1) whose overactivation has been proven to be associated with the pathogenesis of numerous central nervous system disorders (Fig. S2B). Under oxidative stress conditions (e.g. H_2O_2 treatment) PARP-1 activity increases, leading to an accumulation of ADP-ribose (Fig. S2C). Therefore, it is obvious that the medium cultured with neuron pretreated with H_2O_2 can specifically promote neuronal cell death (Fig. S2D).

ADP-ribose triggers GSH-independent ferroptosis

During development and in adulthood, damaged or unwanted cells are eliminated through the activation of different regulated cell death pathways, including apoptosis, necroptosis, pyroptosis, parthanatos and ferroptosis pathways, which have been linked to pathological cell death in the nervous system. In order to determine how the cell death pathway was mediated by ADP-ribose, we used inhibitors of different death pathways to rescue the ADP-ribose-mediated cell death (Fig. 2A). As a result, we found that ferroptosis is the main pathway triggered by ADP-ribose (Fig. 2A). 4-HNE is a well-known by-product of lipid peroxidation and is widely recognized as a stable marker for ferroptosis. The significant up-regulation of 4-HNE under ADP-ribose stress conditions and the reversal of this phenomenon by ferroptosis inhibitor, DFO, forcefully prove that ADP-ribose mediates the ferroptosis pathway (Fig. 2B).

The classic ferroptosis is triggered by the inactivation of cellular glutathione (GSH)-dependent antioxidant defenses, leading to the accumulation of toxic lipid ROS (L-ROS)(*37*). However, ADP-ribose, unlike classic ferroptosis inducers (e.g., glutamate, erastin), does not depend on GSH depletion to trigger ferroptosis (Fig. S3A). The development of metabolomics provides a basis for us to explore the metabolic flow driven by ADP-ribose. We have found that large amount of superoxide accumulates in cells in response to the external ADP-ribose stress, such as hypoxanthine increased by 90 times (Fig. 2C). Hypoxanthine is degraded by enzyme XO and produces ROS, which is the main source of lipid oxidation. When the inhibitor allopurinol or interference shRNA of XO is used to inhibit the degradation of hypoxanthine in cells, the production of ROS triggered by ADP-ribose is significantly reduced (Fig. 2D). In line with it,

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when blocking the production of hypoxanthine through inhibition or depletion of PNP that catalyzes the conversion of inosine to hypoxanthine, the production of 4-HNE is significantly inhibited (Fig. S3B).

Although hypoxanthine is indispensable in ADP-ribose mediated ferroptosis, it is not the only factor, because the inhibition of either PNP or XO can only partially rescue ADP-ribose mediated ferroptosis (Fig. 2D and S3A). For this reason, we speculate that there is another way besides hypoxanthine to trigger ADP-ribose mediated ferroptosis. Through in-depth analysis of the metabolic flux, we found another major metabolic pathway relies on Quinone in mitochondria is indispensable in ADP-ribose mediated ferroptosis (Fig. 2E). The extracellular ADP-ribose stress leads to a large accumulation of glutamine in the cell (Fig. S3C). Subsequently, glutamine is catalyzed by the CAD enzyme to produce a dihydroorotate (Fig. 2E). Dihydroorotate is translocated to mitochondria and converted into orotate under the DHODH activity with the production of ROS(*38*) (Fig. S3D), and these ROS undergo Fenton reaction with the participation of divalent iron ions, resulting in the accumulation of a large number of ROS to promote lipid oxidation (Fig. 2E).

It is obvious that the accumulation of glutamine is the starting point of this pathway. However, most of the glutamine in the cells needs to be absorbed from the environment. Therefore, when we remove the supply of glutamine in the culture medium, we found that even in the presence of ADP-ribose, the downstream dihydroorotate production is also obviously blocked (Fig. 2E). When we calculate the cell survival rate, we find that ADP-ribose can effectively promote cell death when the medium contains glutamine, and this phenotype can be partially rescued by treatment of glutamine transporter inhibiter, i.e., GPNA (Fig. 2F and S3E). However, the lack of glutamine in the culture medium can inhibit ADP-ribose-mediated cell death (Fig. 2F and S3E). It is worth mentioning that the accumulation of glutamine is not unique to the ADP-ribose-triggered ferroptosis, because the treatment of classic ferroptosis inducers, such as glutamate and erastin, can also promote the large accumulation of intracellular glutamine (Fig. S3C).

Taking into account the synergistic contributions of hypoxanthine and dihydroorotate pathways on ADP-ribose mediated ferroptosis, we found that ferroptosis can be almost completely rescued when these two pathways are inhibited at the same time, whether by blocking glutamine intake and inosine degradation (Fig. 2F), or by inhibiting or depleting XO and DHODH (Fig. 2G).

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Interestingly, isoprenoid biosynthesis is essential for all living organisms, which is synthesized by two independent pathways: the MVA and MEP pathways. Our results suggested that these two pathways were inhibited when the cell was stimulated by external ADP-ribose (Fig. S3, F and G).

Comparing the metabolic fluxes produced by ADP-ribose to other ferroptosis inducers, we found that ADP-ribose had similar metabolic spectrum changes with glutamate and erastin, but H2O2 had a relatively independent variation spectrum (Fig. 3A). When the cell responded to the ADP-ribose in the environment, the metabolic pathways of purine, pyrimidine and glutamate were rewiring, which was similar to the effect of glutamate stress (Fig. S4). However, glutamate stress has a bias in the regulation of glutathione metabolism, while ADP-ribose stress is more biased towards the quinone biosynthesis, TCA cycle and glycolysis, which suggest that ADP-ribose-driven ferroptosis has both a mechanism similar to that of traditional inducers and its particularity (Fig. S4).

ADP-ribose inhibits the Activity of ENT1

Considering ADP-ribose and classic ferroptosis inducers result in similar metabolic alternations, we speculate that ADP-ribose may also act on system X_c^-, which is the target of the classic ferroptosis inducer erastin and glutamate. System X_c^- is a disulfide-linked heterodimer composed of SLC7A11 (xCT) and SLC3A2 (Fig. S5A). Consistent with the finding that inhibition of system X_c^- can lead to a compensatory transcriptional upregulation of SLC7A11, we observed substantial upregulation of SLC7A11 in SH-SY5Y cells that were treated with erastin. However, ADP-ribose failed to induce the upregulation of SLC7A11 (Fig. S5B). Furthermore, *in vitro* small molecules-protein binding test shows ADP-ribose does not bind to SLC7A11 directly. (Fig. S5B and 3F). Finally, we directly examined the uptake of [13C]-cystine into SH-SY5Y. Erastin (10 mM) and glutamate (50 mM) abolished the uptake of [13C]-cystine, whereas ADP-ribose had no effect on this process (Fig. S5C). Taken together, these results indicate that system X_c^- is not the direct target of ADP-ribose.

Every cloud has a silver lining. When carefully analyzed the changed major small metabolic molecules in the cells responded to ADP-ribose treatment, we found that the inosine, deoxyinosine and hypoxanthine accumulated in a large amount (Fig. 3B), while the cytidine,

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adenosine and uridine were severely down-regulated (Fig. 3C), which led us to speculate about nucleoside transporters playing important roles in ADP-ribose-mediated ferroptosis. Nucleoside transporters are required as nucleoside that cannot be synthesized *de novo* and are recycled/salvaged. Furthermore, since nucleosides function as second messengers, regulation of their levels is critical for proper neuronal functioning. Nucleoside transporters are classified according to their Na^+ dependence. Equilibrative nucleoside transporters (ENTs) are Na^+-independent and are members of the SLC29A transporter family, whereas concentrative nucleoside transporters (CNTs) are Na^+-dependent and are members of the SLC28A transporter family(*39*). For this reason, we carried out a screening of nucleoside transporters, and found that ADP-ribose stress applied in the environment can lead to a substantial compensatory transcriptional upregulation of ENT1 (Fig. 3D). Consistent with this observation, further analysis of the three-dimensional structural docking reconstruction of the ENT1 in complex with the ADP-ribose suggests that the nucleoside transport pocket is completely blocked by ADP-ribose (Fig. 3E and S5D). Furthermore, ENT1 is identified as a novel binding protein of ADP-ribose using a small-molecule target identification strategy termed drug affinity responsive target stability (DARTS) (Fig. 3F) and a cellular thermal shift assay for evaluating drug target interaction in cells (Fig. S5E). Given these results, we directly examined the uptake of cytidine and adenosine into SH-SY5Y cells, and found that ADP-ribose abolished the uptake of cystine and adenosine (Fig. 3G). There is a general agreement that the purine nucleoside adenosine is an important neuromodulator, which appears to arise primarily by inhibition of release of transmitters, including glutamate. We found that siRNA-mediated silencing of ENT1 with two independent siRNAs induced glutamate enrichment in SH-SY5Y cells, consistent with this, ADP-ribose or adenosine treatment had similar effects on this process (Fig. 3H and S5F).

siRNA-mediated silencing of ENT1 sensitized SH-SY5Y cells to ADP-ribose-induced cell death, which is further confirmed by the relevance of ENT1 to ADP-ribose-induced ferroptosis (Fig. 3I and S5F). Cultures of the SH-SY5Y cell line exposed to retinoic acid (RA) to induce neuronal differentiation that demonstrated low proliferative rates and a pronounced neuronal morphology (Fig. S5G), have low expression of ENT1 (Fig. S5H). Compared with undifferentiated cells, RA differentiated HN cells are more sensitive to the neurotoxic effect of ADP-ribose (Fig. 3J).

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In summary, the ADP-ribose that was secreted to the extracellular matrix or was released into the environment by cell death binds to the ENT1 on the surface of neuronal cells to inhibit the import of extracellular cytidine, adenosine or uridine, and the export of intracellular ROS precursors, such as hypoxanthine and inosine. The former promotes the synthesis of glutamine into lipids and dihydroorotate production by inhibiting the release of intracellular glutamate, while the latter combines produced dihydroorotate to produce too much ROS, which mediates lipid peroxidation to trigger ferroptosis (Fig. 3K). Those two intersecting pathways together determine the ADP-ribose-mediated ferroptosis.

Intracellular ADP-ribose is an accelerator of ferroptosis

Based on the principle that proteins become more resistant to heat-induced unfolding when complexed with a ligand, thermal proteome profiling (TPP) allows for an unbiased search of drug targets(*40*). Through TPP systematic screening coupled with mass spectrometry (Fig. S6A), yet we still found that 42 out of the 7326 effective proteins can be stabilized by ADP-ribose (Fig. 4A). In mammals, NUDIX hydrolases (NUDTs) exhibit a wide range of functions and are characterized by different substrate specificity and intracellular localization. In our database, we found that only NUDT9 and NUDT14 of the 17 detected NUDTs have the ability to bind to ADP-ribose, which is consistent with previously published reports(*41*) and confirms the reliability of our thermal profiling data (Fig. S6, B and C).

GO analyses of molecular functions of proteins increased thermodynamic stability yielded a group of NAD/NADP binding proteins that have oxidoreductase activity (Fig. 4B). We found that most of ADP-ribose binding proteins use NAD/NADP as a coenzyme factor and have the classical catalytic activity (e.g., GAPDH and ADH5, Fig. S6, D and E). However, it is incredible that the enzymes with NAD as the co-substrate (e.g., PARP1 and Sirt1) have no direct binding ability with ADP-ribose (Fig. S6, F and G). So, we speculated that NAD uses the ADP-ribose-end to contact the former that adds a hydrogen ion to the nicotinamide (NAM)-end to form reduced NADH, but the later binds to the nicotinamide-end of NAD and transfers the ADP-ribose moiety to target proteins, releasing nicotinamide. Furthermore, a further analysis revealed that the targets of ADP-ribose could be roughly divided into five main types. The first category is the previously mentioned NAD-dependent enzymes (NAD as a coenzyme). The second is ADP-ribose glycohydrolase (e.g. ADPRS) and ADP-ribose pyrophosphatase (e.g. NUDT9/14),

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which are involved in the formation or hydrolysis of ADP-ribose monomers. The third is ADP/ATP- dependent enzymes (e.g. AK4, Fig.S6I), where it is possible that the ADP/ATP binding pocket of some enzymes can accommodate ADP-ribose or its ADP-end positions. The fourth is non-substrate similarity enzymes, in which CS and AP2M1/SNX21 are representative (Fig.S6, H and J). Citrate synthase (CS), as a pace-making enzyme in the first step of the citric acid cycle, catalyzes the condensation reaction between acetyl coenzyme A and oxaloacetate to form citrate. It plays an important role in ferroptosis. However, AP2M1 and SNX21 participate in lipid binding. Last but not the least there is the enzymes of unknown function, such as C11orf57 and C16orf80 (Fig.S6K). Moreover, in order to verify the reliability of the data, we tested the effect of ADP-ribose on the thermostability (Fig. S6L) and enzyme activity (Fig. S6M) of GAPDH and CS. Finally, we found that ADP-ribose can directly bind to GAPDH and CS to inhibit their activities (Fig. S6, L and M).

Interestingly, in the NAD^+-coenzyme dependent protein cluster we found that ADP-ribose is partial to the aldehyde dehydrogenases (ALDHs) that are involved in the detoxification of both exogenous and endogenous aldehydes (Fig. 4C). ALDH7A1, an important paralog of ALDH1B1, protects cells from oxidative stress by metabolizing a number of lipid peroxidation–derived aldehydes(*42*). The cellular thermal shift assay shows the specific interaction between ALDH7A1 and ADP-ribose, but no ADP-ribose premise, PAR, or degradation product, R5P (Fig. 4, D and E). Furthermore, dehydrogenase activity analyses of ALDH7A1 proteins indicate that ADP-ribose strongly inhibits the ALDH7A1 activity (Fig. 4F). Moreover, up-regulation of 4-HNE induced by glutamate can sharply increase under the treatment of dehydrogenase inhibitor, disulfiram (Fig. 4H). Consistent with this, disulfiram treatment exacerbated cell death induced by glutamate (Fig. 4, I and J). In addition, shRNA-mediated stable silencing of ALDH7A1 induced the up-regulation of 4-HNE in SH-SY5Y cells treated with glutamate, but not ADP-ribose (Fig. 4G). These results suggest that ALDH7A1 are important targets of ADP-ribose and ALDH7A1 inhibition by ADP-ribose cause a sharp accumulation of 4-HNE to exacerbate cell death.

ADP-ribose is the therapeutic target

Poly (ADP-ribose) (PAR) is the main source of ADP-ribose in cells. We found that glutamate can trigger PAR production in cells and PARG inhibitor, PDD, can induce PAR accumulation (Fig. 5A and S7, A and B). iRucaparib-AP6 is a PROTAC PARP1 degrader (PPD) developed in

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our Lab previously(*43*), which can effectively inhibit the production and accumulation of PAR in SH-SY5Y cells induced by glutamate treatment (Fig. 5A and S7, A and B). Coincidentally, erastin can more effectively induce the production of PAR, which still relies on PARP1, because PPD can effectively inhibit the PAR accumulation (Fig. 5A and S7, A and B). These results demonstrated that the classic ferroptosis inducers could trigger the destruction of cell redox homeostasis and activate PARP1, and a large amount of PAR produced was further decomposed into mono-ADP-ribose.

Considering the source of ADP-ribose, we decided to block the cell death induced by ADP-ribose from the following three aspects. Firstly, inhibitors were used to block the production of ROS induced by ADP-ribose in cells; Secondly the small molecule drug PPD was applied to block the production of ADP-ribose precursor, PAR; and finally, the enzyme NUDT9 were engineered to deplete the extracellular ADP-ribose (Fig. S7C). We found that both allopurinol/atovaquone (iROS) and PPD treatment can effectively rescue glutamate-induced neuronal cell death (Fig. 5B and S7C). Assuredly, NUDT9 treatment has similar effects with ROS inhibitors treatment (Fig. 5B and S7C).

Based on the genetic similarity (including XO and DHOD) and similar organs and biological processes with mammals, *Drosophila* provides a good background for genetic and biologic studies of neurological disorders. Fruit flies affected by neurodegeneration share behavioral defects and reduced lifespans, which is evidenced by the shortened lifespan of glutamate-fed fruit flies (Fig. S7D). As expected, ADP-ribose treatment significantly induced wing defects of *Drosophila*, meanwhile, the flies climbing ability were severely downgraded under ADP-ribose treatment (Fig. 5, C-E). Furthermore, ADP-ribose can promote climbing abnormalities caused by glutamate feeding (Fig. S7E). Moreover, the survival of ADP-ribose-fed fruit flies is significantly reduced, but adding ROS inhibitors, allopurinol and atovaquone, to food will significantly improve the lifespan shortened by ADP-ribose treatment (Fig. 5F).

Overexpression of mutant tau (tau^{R406W}) in the fly brain results in a progressive neurodegenerative phenotype characterized by histological abnormalities, including degeneration of neurons in the cortex and neuropil vacuolization(*44*). We found for the first time that the content of ADP-ribose in the brain of mutant fruit flies was significantly higher than that of wild-type fruit flies (Fig. 5G). Meanwhile, compared with wild-type fruit flies, we found that the

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dehydrogenase activity in the brain of mutant fruit flies was significantly inhibited, which is consistent with the wild-type fruit flies fed with glutamate (Fig. S7F). Fortunately, the use of allopurinol and atovaquone effectively alleviated the surviving restriction of mutant fruit flies (Fig. 5G).

Discussion

Endogenous metabolic small molecules that trigger nerve cell death

Neuronal cell death occurs extensively during development and pathology, which is especially important because of the limited regeneration capacity of adult neurons. Disruption of cellular functions with an aging-induced accumulation of neuronal stressors causes cell death which is a common feature of neurodegenerative diseases. Small molecules currently known are closely related to neurological diseases, mainly including PAR, glutamate, ROS, calcium and iron. Here, to the best of our knowledge, it is the first time that ADP-ribose is propose to be an endogenous metabolic small molecule that triggers the nerve cell death through a novel mechanism, which greatly deepens our understanding of nervous system diseases induced by nerve cell death.

Poly (ADP-ribose) (PAR)-dependent cell death, also named parthanatos, is responsible for neuronal loss in neurological diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS). Parthanatos has distinct features that differ from caspase-dependent apoptosis, necrosis or autophagic cell death. Parthanatos can be triggered by the accumulation of PAR due to the overactivation of PAR polymerase-1 (PARP-1). Excess PAR, induces the mitochondrial release apoptosis-inducing factor (AIF), which binds to macrophage migration inhibitory factor (MIF) carrying MIF into the nucleus where it cleaves genomic DNA into large fragments(*45-47*). Excessive extracellular glutamate may lead to excitotoxicity *in vitro* and *in vivo* in acute insults like ischemic stroke via the overactivation of ionotropic glutamate receptors. In addition, chronic excitotoxicity has been hypothesized to play a role in numerous neurodegenerative diseases, which is probably related to glutamate-mediated ferroptosis(*48*). Although we found that glutamate can also induce the accumulation of ADP-ribose, ferroptosis induced by ADP-ribose and glutamate relies on two different independent pathways.

ADP-ribose triggers GSH-independent ferroptosis

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In a recent study, Bersuker et al. used a synthetic lethal CRISPR-Cas9 screening to confirme that FSP1, formerly known as AIFM2, was an effective ferroptosis-resistance factor. Furthermore, Doll et al. also performed relevant research on FSP1. An expression cloning method was used to identify genes in human cancer cells that complement the loss of GPX4. Further studies have shown that the myristoylation of FSP1 mediates the recruitment of this protein to the plasma membrane, where it performs its function as an oxidoreductase that reduces COQ10 (also known as ubiquinone-10) and as a lipophilic radical-trapping antioxidant that halts the propagation of lipid peroxides. Doll et al. showed that FSP1 catalyzes the regeneration of COQ10 by NAD(P)H, and the FSP1-CoQ10-NAD(P)H pathway is an independent parallel system that cooperates with GPX4 and glutathione to suppress phospholipid peroxidation and ferroptosis. Moreover, this also explains the effect of NAD(P)H in the MVA pathway through the loss of ubiquinone convergence on FSP1 and thereby predicts sensitivity to ferroptosis(*49, 50*).

Our data shows that ADP-ribose triggered ferroptosis depends on the metabolism of purine and pyrimidine. The accumulation of a large number of purines with oxidative potential in neuron provides the conditions for the tamping of ROS production and lipid peroxidation, while the supersaturation of the pyrimidine synthesis leads to the same events. DHODH located on the outer surface of the inner mitochondrial membrane (IMM), catalyze the ubiquinone-mediated oxidation of dihydroorotate to orotate, in de novo pyrimidine biosynthesis, which is accompanied by the ROS production. In fact, the glutamine-dihydroorotate-quinone pathway and FSP1-CoQ10-NAD(P)H pathway are opposite. The former is the consumption of ubiquinone while the latter is the regeneration of ubiquinone, thus determining the promoting and inhibiting effects of these two pathways in the process of ferroptosis.

ADP-ribose inhibits the Activity of ENT1

Human Nucleoside Transporters (hNTs) belong to two different gene families, SLC28 and SLC29, encoding human Concentrative Nucleoside Transporters (hCNTs) and human Equilibrative Nucleoside Transporters (hENTs), respectively. hCNTs and hENTs are integral membrane proteins, albeit structurally unrelated. Both families share common features as substrate selectivity and often tissue localization. This apparent biological redundancy may anticipate some different roles for hCNTs and hENTs in cell physiology. Thus, hENTs may have a major role in maintaining nucleoside homeostasis, whereas hCNTs could contribute to

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nucleoside sensing and signal transduction. Therefore the ascription of hCNT1 to a transceptor reinforces this hypothesis.

In humans ENTs are also known as SLC29, a group of plasmalemmal transport proteins which transport nucleoside substrates like adenosine into cells. There are four known human ENTs, designated ENT1, ENT2, ENT3, and ENT4. They are blocked by adenosine reuptake inhibitors like dipyridamole and dilazep, drugs used clinically for their vasodilatory properties. The best-characterized members of the human Ent family, hENT1 and hENT2, possess similar broad permeant selectivities for purine and pyrimidine nucleosides, but hENT2 also efficiently transports nucleobases. hENT3 has a similar broad permeant selectivity for nucleosides and nucleobases and appears to function in intracellular membranes, including lysosomes. Gemcitabine, an anti-cancer drug, is transported by hENT1 and hENT3. hENT4 is uniquely selective for adenosine, and also transports a variety of organic cations. Regarding CNS, animal models and patient samples have revealed that ENT1 and adenosine constitute biomarkers of the initial stages of neurodegeneration in Huntington's disease with ENT1 transcript being significantly upregulated in those patients(*39*).

In the human brains, protein expression of ENT1 was found to be predominant in the frontal and parietal lobes of the cerebral cortex, thalamus, midbrain, and basal ganglia. In mixed cultures of chick retina cells, more than 90% of [3H]-adenosine taken up by cells is converted into adenine nucleotides, while around 80% of purine release stimulated by activation of ionotropic glutamate receptors is found as inosine and hypoxanthine. Interestingly, similar results were previously described in purified cultures of chick retinal neurons. This stimulatory effect on purine release induced by glutamate is blocked by the ENT1 blocker NBMPR. These results are similar in some terms with a study made by Perez and colleagues, who demonstrated that the major amount of [3H]-adenosine taken up by rabbit retina cells is converted into adenine nucleotides and, in the presence of a depolarizing stimulus, an increase of purine release is observed, mainly as hypoxanthine, xanthine, and inosine. This release was partially blocked by dipyridamole, an inhibitor of nucleoside transporters(*51-53*).

Here, we found that ADP-ribose, as a natural inhibitor of ENT1, blocked the adenosine uptake and purine release. ADP-ribose enters the nucleoside transport pocket of ENT1, where ADP-ribose interacts with multiple amino acids of ENT1, such asT35, Q37, M33/84, P308/330,

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leading to the seal of this pocket and the loss of the nucleoside transport ability of ENT1. This directly leads to the accumulation of a lot of adenosine outside the cell which inhibits the release of glutamate and the accumulation of purines inside the cell which leads to the increase of ROS and lipid oxidation. In fact, the correlation between ADP-ribose and adenosine can be easily found from the molecular structure. ADP-ribose is adenosine with ribose 5'-biphosphate (R5BP). When the adenosine terminal of ADP-ribose enters the nucleoside transport pocket, ADP-ribose stays in the pocket and blocks the normal nucleoside transport due to the interaction between the R5BP terminal and amino acids on ENT1.

Intracellular ADP-ribose targets

NAD-cofactor enzymes are identified as a novel binding protein of ADP-ribose through the small-molecule target identification strategies termed thermal proteome profiling (TPP) and drug affinity responsive target stability (DARTS), but NAD-consuming enzymes are not. This interesting phenominon reminds us the both enzymes use NAD, yet they bind to NAD in a different way. The former supports a redox reaction but the latter mediates by removing a nicotinamide. Therefore, the NAD binding pockets of these two enzymes are biased towards the combination of ADP-ribose and nicotinamide terminals, respectively.

ALDHs, as the NAD-cofactor enzymes, can be bound and regulated by ADP-ribose. ALDHs located at different subcellular compartments are able to detoxify a wide variety of aldehydes to their corresponding carboxylic acids, thus facilitating to protect them from oxidative stress. Many different diseases are associated with oxidative stress. Lipid peroxidation is the process by which oxygen combines with lipids to generate lipid hydroperoxides via intermediate formation of peroxyl radicals. One of the main consequences of oxidative stress at the cellular level is lipid peroxidation, where toxic aldehydes may be generated. Below the tolerance of toxicity thresholds, some aldehydes are involved in signaling processes, while others are intermediaries in the metabolism of lipids, amino acids, neurotransmitters, and carbohydrates. In contrast, some aldehydes ubiquitously distributed in the environment, such as acrolein or formaldehyde, are extremely toxic to the cell. Ferroptosis is an iron-dependent oxidative form of cell death associated with increased lipid peroxidation and insufficient capacity to eliminate lipid peroxides.

ADP-ribose is the therapeutic target

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The results of ADP-ribose microinjection in the cerebral cortex and hippocampus of mice and drug stimulation of primary neurons demonstrate that ADP-ribose has a significant lethal effect on neurons. Effective inhibition of the damaging effect of ADP-ribose on nerve cells can provide prospective guidance for the therapy of neurodegenerative diseases. This goal can be achieved from at least two aspects: inhibiting NAD-consuming enzymes to control the source of ADP-ribose, and specifically inhibiting the generation of ROS triggered by ADP-ribose.

As we discussed earlier, all NAD-consuming enzymes lead to the formation of nicotinamide, so in theory nicotinamide is their natural inhibitor, which is also verified on SIRTuins and PARPs etc. Nicotinamide, the amide form of vitamin B3 (niacin), appears to play a role in protecting neurons from traumatic injury, ischaemia, and stroke, as well as being implicated in 3 key neurodegenerative conditions: Alzheimer's, Parkinson's, and Huntington's diseases. However, a key factor is the bioavailability of nicotinamide, with low concentrations of which leading to neurological deficits and dementia while high levels of which potentially causing neurotoxicity(*11*, *54*). Therefore, the development of specific NAD-consuming enzyme inhibitors is necessary. Meanwhile, proteolysis targeting chimera (PROTAC) provides unprecedented convenience for this. We found that the PARlation level increased significantly under the treatment of glutamate and erastin. Based on the analysis above, we use a newly developed PARP1 degradable small molecule inhibitor, named PPD, to induce the degradation of endogenous PARP1. We found that the PPD treatment can effectively inhibit nerve cell death induced by glutamate excitotoxicity. What's more, when added ADP-ribose hydrolase NUDT9 to the culture medium, we found that the nerve cell death induced by glutamate was effectively inhibited, which indicated the negative effect of extracellular ADP-ribose on nerve cells and the expectation of development of engineered enzymes to solve the diseases triggered by ADP-ribose as well.

In addition to the consideration of enzyme genes, we have also tried new use of old drugs based on the pathogenic mechanism of ADP-ribose. Several studies performed on various cellular or animal models of tauopathies have established that overexpression of mutant forms of human tau underlying various types of dominantly inherited tauopathies increases both the expression of oxidative stress (OS) markers and the sensitivity of neurons to oxidant molecules. ROS production is a required signal for PARP-1 activation in neurons. The R406W tau mutation is a

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unique missense mutation whose patients have been reported to exhibit Alzheimer's disease (AD)-like phenotypes rather than the more typical familial frontotemporal dementia (FTD) phenotypes. *UAS-R406W tau/+* transgenic flies show neurodegeneration and die prematurely. Compared with wild-type flies, the ADP-ribose content in the brains of transgenic flies is significantly higher.

Since ADP-ribose inhibits ENT1 activity, a large amount of hypoxanthine (about 90 folds) was accumulated in the cell. Xanthine oxidase (XO) catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid, which generates reactive oxygen species (ROS). This is one of the main reasons that ADP-ribose induces ROS production. Purine analogues allopurinol, a xanthine oxidase inhibitor, is indicated for reducing the production of uric acid and treatment of hyperuricemia, gout and reperfusion injury. But here we found that it can also reduce ADP-ribose's damage to nerve cells. The other way is caused by the massive intake of glutamine induced by ADP-ribose. Glutamine (Gln) abounds in the central nervous system (CNS), and its interstitial and cerebrospinal fluid (CSF) concentrations are at least one order of magnitude higher than of any other amino acid. Gln transport from blood to the brain is insufficient to meet the demand of the brain tissues for this amino acid. The "glutamate-glutamine" cycle is the key to compensate for the lack of glutamine, in which Glu released from neurons is taken up by astrocytes, and reconverted to Gln then shuttled to neurons. But the excessive accumulation of Gln in brain cells may be deleterious to brain function. The inhibition of ADP-ribose on ENT1 leads to the accumulation of extracellular adenosine and thus inhibits the glutamate release, which leads to paralysis of the "glutamate-glutamine" circulatory system and continuing glutamine uptake of neurons from the surrounding environment. In addition to synthesizing lipid through the α-KG-Ac-CoA pathway, excess glutamine also produces a large amount of peroxide, dihydroorotate, through the CAD pathway. Dihydroorotate dehydrogenase (DHODH) catalyzes the ubiquinone (e.g. CoQ10)-mediated oxidation of dihydroorotate to orotate and generates active oxygen. Although it is only known that inhibitors of this enzyme are used to treat autoimmune diseases such as rheumatoid arthritis, here we found that atovaquone, a DHODH inhibitor, has an important contribution to save ADP-ribose-induced neuronal death. Taken together, ADP-ribose may be an attractive therapeutic target for the treatment of acute neuronal damage and possibly neurodegenerative diseases.

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Fig.1 ADP-ribose is a neuronal death factor

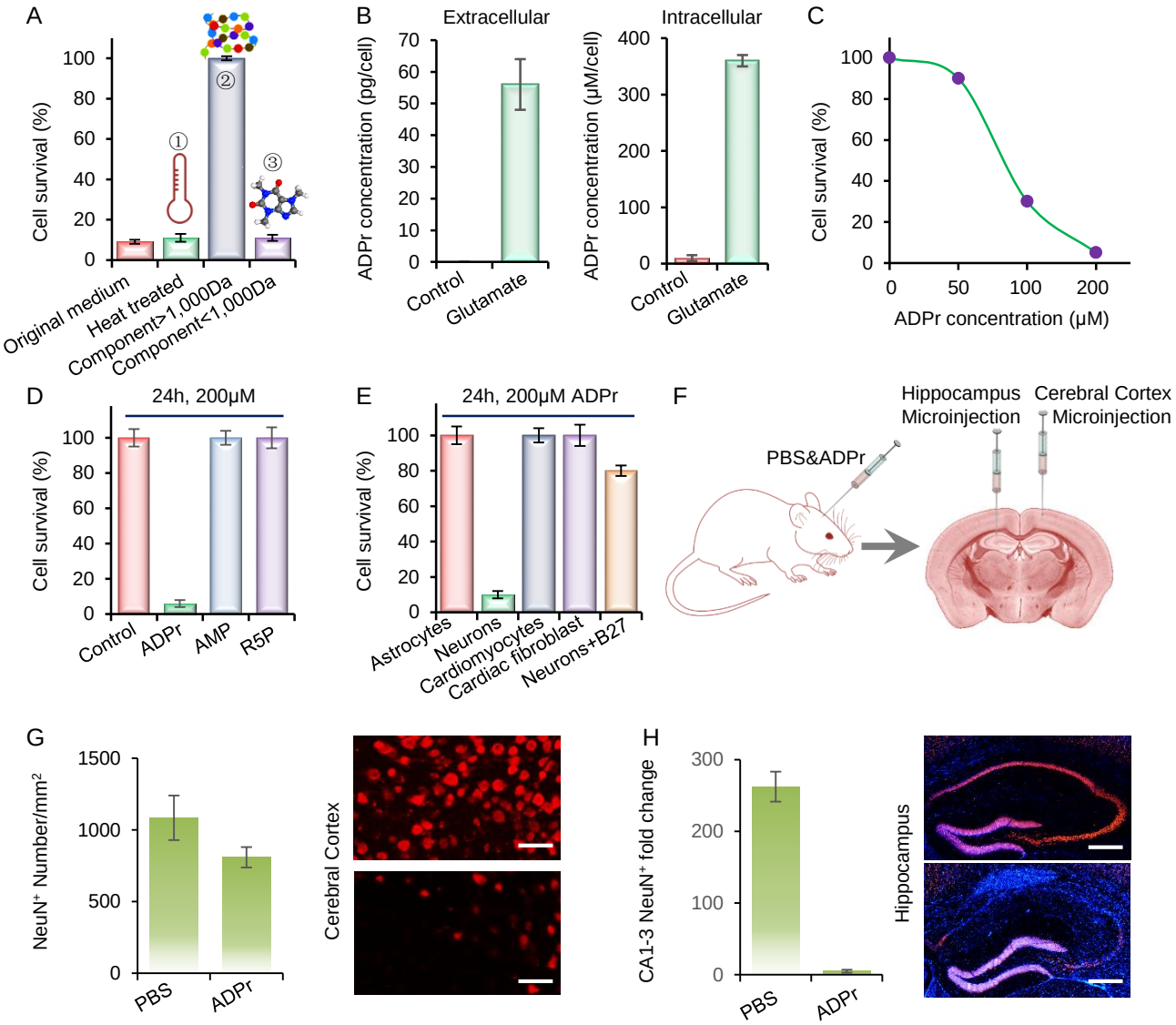

Figure 1. ADP-ribose is a neuronal death factor

(A) The neurons obtained from EMX-A14 transgenic mice were treated with 5 mM glutamate for 6 hours and then replaced by the fresh medium to continue culturing for 6 hours, the collected medium was followed by different treatment then added to the neuron cell culture medium. The cell viability was tested after 24 hours.

(B) QTrap 6500 was used to construct a standard measuring curve (ADP-ribose from Sigma, A0752) to measure extracellular and intracellular ADP-ribose concentration under glutamate treatment for 12 hours.

(C) Treat neuronal cells with different concentrations of ADP-ribose and the cell viability was tested after 12 hours.

(D) Neuronal cells were exposed to 200μM ADP-ribose, AMP and R5P. The cell viability was tested after 24 hours.

(E) Different primary cells obtained from mice were exposed to 200μM ADP-ribose, and the cell viability was tested after 24 hours. The high amount of antioxidants present in the neuronal cell culture supplement B27.

(F) The schematic diagram shows the hippocampus microinjection and cerebral cortex microinjection to evaluate the role of ADP-ribose *in vivo*.

(G) Characterizing the damage of ADP-ribose to cerebral cortex neurons by counting the NeuN-positive cells. Representative microscopic fluorescence imaging describes the death-promoting effect of ADP-ribose on cerebral cortex neurons.

(H) The histogram shows the change of neurons number in CA1-3 areas by counting the NeuN-positive cells. Representative microscopic fluorescence imaging describes the death-promoting effect of ADP-ribose on hippocampus neurons.

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Fig.2 ADP-ribose triggers GSH-independent ferroptosis

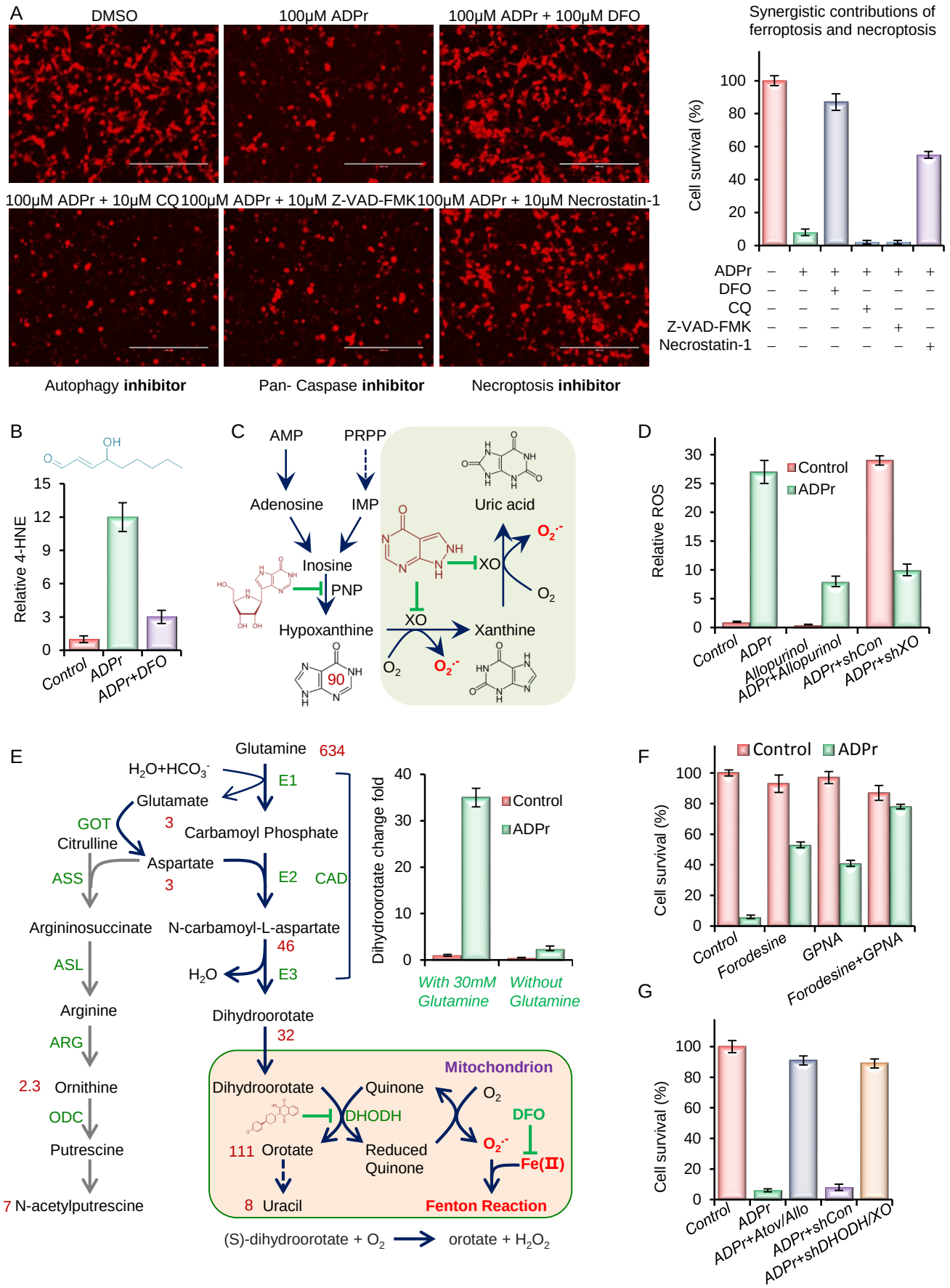

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Figure 2. ADP-ribose triggers GSH-independent ferroptosis

(A) The inhibitors of different death pathways were used to rescue the ADP-ribose-mediated neuronal cell death. The concentration of different compounds is indicated in the picture, and the processing time is 12 hours. Representative microscopic fluorescence imaging is on the left and the histogram describes cell viability on the right.

(B) 4-HNE, as a stable marker for ferroptosis, is up-regulated under ADP-ribose stress conditions. The ferroptosis inhibitor, DFO, forcefully prove that ADP-ribose mediates the ferroptosis pathway.

(C) The hypoxanthine-XO-uric acid pathway is indispensable in ADP-ribose mediated ferroptosis.

(D) The inhibition of either PNP or XO can partially rescue ADP-ribose mediated ferroptosis.

(E) The glutamine-dihydroorotate-quinone pathway is important in ADP-ribose mediated ferroptosis. Glutamine uptake is indispensable for ADP-ribose mediated ferroptosis to produce dihydroorotate.

(F) ADP-ribose can effectively promote cell death when the medium contains glutamine, and this phenotype can be partially rescued by treatment of glutamine transporter inhibiter, i.e., GPNA. Forodesine, PNP inhibitor.

(G) Ferroptosis can be almost completely rescued by inhibiting or depleting XO and DHODH. Allopurinol, XO inhibitor; Atovaquone, DHODH inhibitor.

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Fig.3 ADP-ribose inhibits the Activity of ENT1

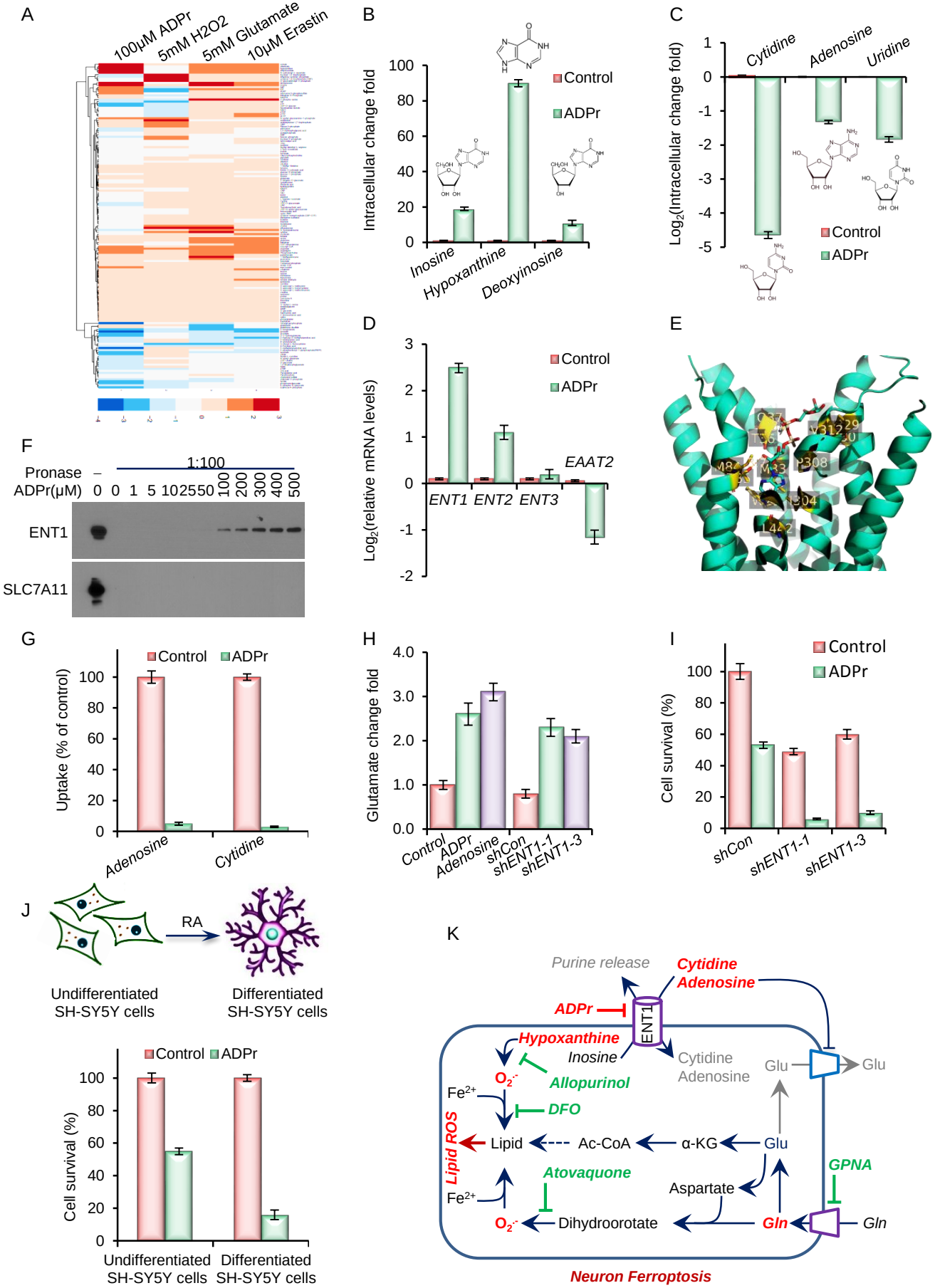

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Figure 3. ADP-ribose inhibits the activity of ENT1

(A) ADP-ribose had similar metabolic spectrum changes with glutamate and erastin, but H2O2 had a relatively independent variation spectrum.

(B) The inosine, deoxyinosine and hypoxanthine accumulated in a large amount in the cells responded to ADP-ribose treatment.

(C) The cytidine, adenosine and uridine were severely down-regulated in the cells responded to ADP-ribose treatment.

(D) ADP-ribose stress applied in the environment can lead to a substantial compensatory transcriptional upregulation of ENT1.

(E) Three-dimensional structural docking reconstruction of the ENT1 in complex with the ADP-ribose shows that the nucleoside transport pocket is completely blocked by ADP-ribose.

(F) ENT1 is identified as a novel binding protein of ADP-ribose using a small-molecule target identification strategy termed drug affinity responsive target stability (DARTS).

(G) ADP-ribose abolished the uptake of cystine and adenosine into SH-SY5Y cells.

(H) siRNA-mediated silencing of ENT1 with two independent siRNAs induced glutamate enrichment in SH-SY5Y cells.

(I) siRNA-mediated silencing of ENT1 sensitized SH-SY5Y cells to ADP-ribose-induced cell death.

(J) Compared with undifferentiated cells, retinoic acid differentiated HN cells are more sensitive to the neurotoxic effect of ADP-ribose.

(K) The ADP-ribose that was secreted to the extracellular matrix or was released into the environment by cell death binds to the ENT1 on the surface of neuronal cells to inhibit the import of extracellular cytidine, adenosine or uridine, and the export of intracellular ROS precursors, such as hypoxanthine and inosine. The former promotes the synthesis of glutamine into lipids and dihydroorotate production by inhibiting the release of intracellular glutamate, while the latter combines produced dihydroorotate to produce too much ROS, which mediates lipid peroxidation to trigger ferroptosis. Those two intersecting pathways together determine the ADP-ribose-mediated ferroptosis.

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Fig.4 Intracellular ADP-ribose is an accelerator of ferroptosis

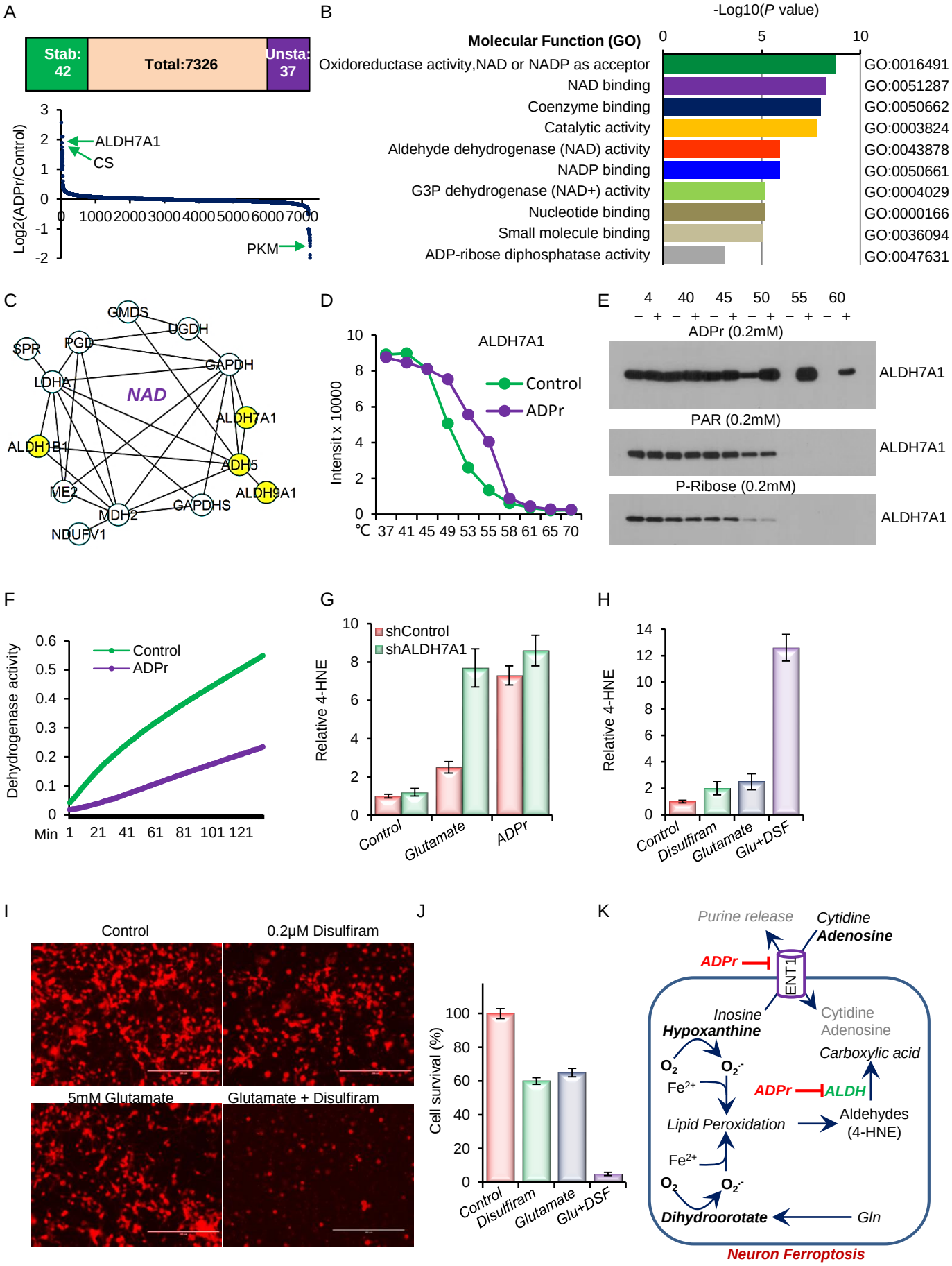

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Figure 4. Intracellular ADP-ribose is an accelerator of ferroptosis

(A) Through thermal proteome profiling (TPP) systematic screening coupled with mass spectrometry shows that 42 out of the 7326 effective proteins can be stabilized by ADP-ribose.

(B) GO analyses of molecular functions of proteins increased thermodynamic stability yielded a group of NAD/NADP binding proteins that have oxidoreductase activity.

(C) ADP-ribose is partial to the aldehyde dehydrogenases (ALDHs) that are involved in the detoxification of both exogenous and endogenous aldehydes.

(D) The cellular thermal shift assay shows the specific interaction between ALDH7A1 and ADP-ribose.

(E) The cellular thermal shift assay shows the specific interaction between ALDH7A1 and ADP-ribose, but no ADP-ribose premise, PAR, or degradation product, R5P.

(F) Dehydrogenase activity analyses of ALDH7A1 proteins indicate that ADP-ribose strongly inhibits the ALDH7A1 activity.

(G) shRNA-mediated stable silencing of ALDH7A1 induced the up-regulation of 4-HNE in SH-SY5Y cells treated with glutamate, but not ADP-ribose.

(H) Up-regulation of 4-HNE induced by glutamate can sharply increase under the treatment of dehydrogenase inhibitor, disulfiram.

(I) Representative microscopic fluorescence imaging describes disulfiram treatment exacerbated neuronal cell death induced by glutamate.

(J) The neuronal cell viability was tested under glutamate and disulfiram treatment after 24 hours.

(K) The dual role of intracellular and extracellular ADP-ribose.

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Fig.5 ADP-ribose is the therapeutic target

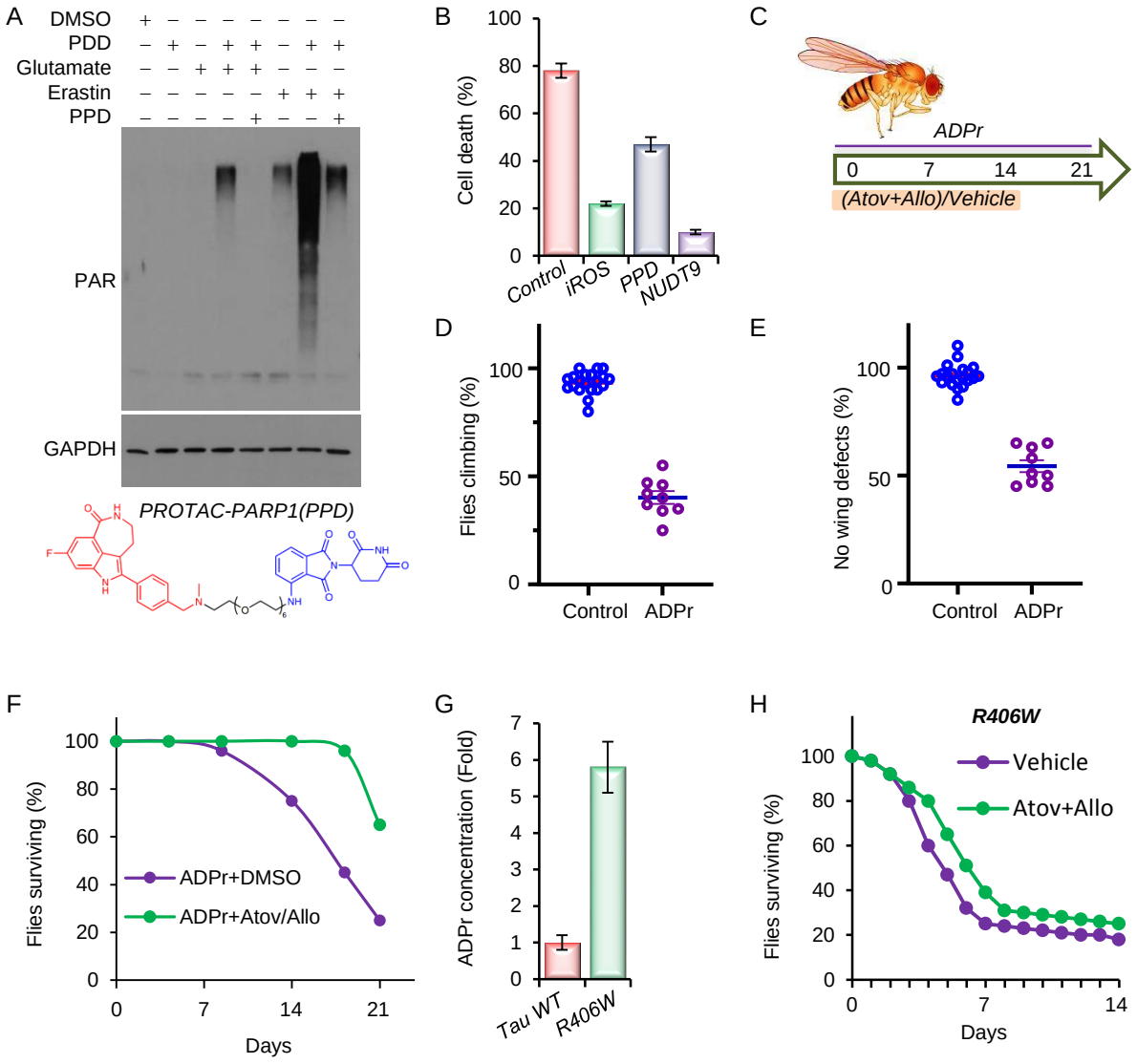

Figure 5. ADP-ribose is the therapeutic target

(A) Glutamate can trigger PAR production in cells and PARG inhibitor, PDD, can induce PAR accumulation. iRucaparib-AP6 is a PROTAC PARP1 degrader (PPD) can effectively inhibit the production and accumulation of PAR in SH-SY5Y cells induced by glutamate treatment.

(B) Allopurinol/atovaquone (iROS) and PPD treatment can effectively rescue glutamate-induced neuronal cell death. Assuredly, NUDT9 treatment has similar effects with ROS inhibitors treatment.

(C) Diagram of *Drosophila* drug treatment.

(D) The flies climbing ability of *Drosophila* were severely downgraded under ADP-ribose treatment.

(E) ADP-ribose treatment significantly induced wing defects of *Drosophila*.

(F) The survival of ADP-ribose-fed fruit flies is significantly reduced, but adding ROS inhibitors, allopurinol and atovaquone, to food will significantly improve the lifespan shortened by ADP-ribose treatment.

(G) The content of ADP-ribose in the brain of tau^{R406W} mutant fruit flies was significantly higher than that of wild-type fruit flies.

(H) The use of allopurinol and atovaquone effectively alleviated the surviving restriction of tau^{R406W} mutant fruit flies.

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Fig.S1 ADP-ribose is a neuronal death factor

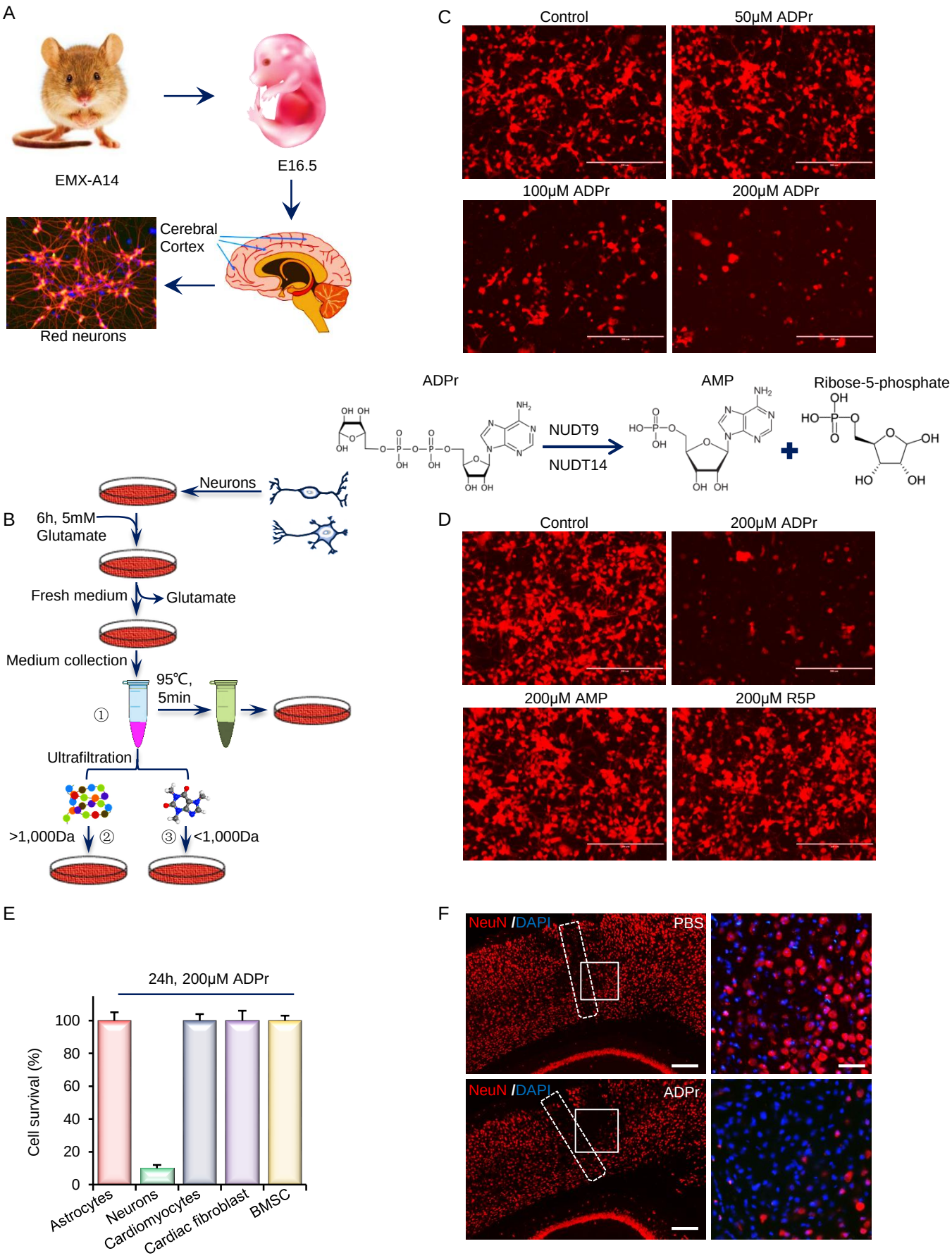

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Figure S1. ADP-ribose is a neuronal death factor

(A) Schematic diagram of the neuron separation workflow. Neuronal cells were obtained from the cerebral cortex of embryonic 16.5 days EMX-A14 transgenic mice, which was labeled with red fluorescent protein (RFP).

(B) Neurons responded to excessive glutamate may release certain factors into the extracellular environment to trigger neurons and adjacent cells death. The neurons were treated with 5 mM glutamate for 6 hours and then replaced by the fresh medium to continue culturing for 6 hours, the collected medium was followed by different treatment then added to the neuron cell culture medium.

(C) Representative microscopic fluorescence imaging describes the death-promoting effect of ADP-ribose on neuronal cells. Treat neuronal cells with different concentrations of ADP-ribose. After 12 hours, count the cell numbers under a fluorescence microscope and calculate the survival rate.

(D) Representative microscopic fluorescence imaging describes the death-promoting effect of AMP and R5P on neuronal cells. Although AMP and R5P come from the ADP-ribose degradation, they had no effect on cell death.

(E) Different types of cells have inconsistent responses to ADP-ribose. Different primary cells obtained from mice were exposed to 200μM ADP-ribose, and the cell viability was tested after 24 hours.

(F) Cerebral cortex microinjection of ADP-ribose was performed in mice. One day after the drug intervention, the mice were executed. The frozen sections of the cerebral cortex were obtained and stained with DAPI for DNA and with NeuN for surviving neuronal cells. The boxed areas by solid line are magnified on the right. The dashed areas specify the needle trajectory. Scale bar: 50μm (main); 12.5μm (magnification).

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Fig.S2 ADP-ribose triggers neuronal cell death

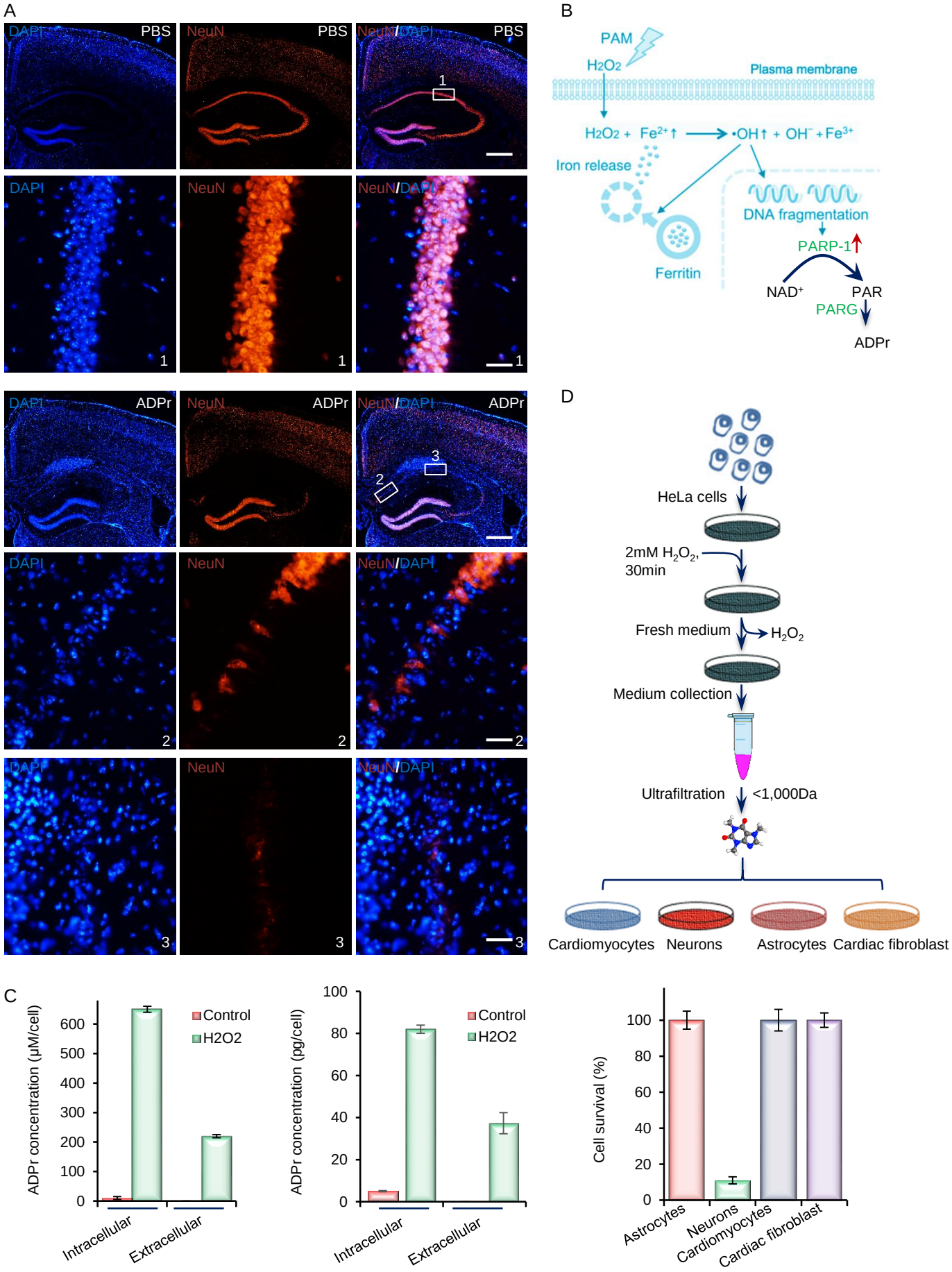

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Figure S2. ADP-ribose triggers neuronal cell death

(A) Hippocampus microinjection of ADP-ribose was performed in mice. One day after the drug intervention, the mice were executed. The frozen sections of the brain were obtained and stained with DAPI for DNA and with NeuN for surviving neuronal cells. The boxed areas are numbered and magnified on the below. Scale bar: 50μm (main); 25μm (magnification).

(B) The schematic diagram shows that the oxidative stress (e.g. hydrogen peroxide treatment) promotes the rapid accumulation of ADP-ribose in the cell through the PARP1 pathway.

(C) QTrap 6500 was used to construct a standard measuring curve (ADP-ribose from Sigma, A0752) to measure the intracellular and extracellular ADP-ribose concentration under hydrogen peroxide treatment.

(D) Neurons responded to excessive glutamate may release certain factors into the extracellular environment to trigger neurons and adjacent cells death. The neurons were treated with 2 mM H2O2 for 30 minutes and then replaced by the fresh medium to continue culturing for 9 hours. The ultrafiltration of collected medium was carried out and the <1,000 Da components were added to the different primary cell culture medium, and the cell viability was tested after 24 hours.

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Fig.S3 ADP-ribose triggers GSH-independent ferroptosis

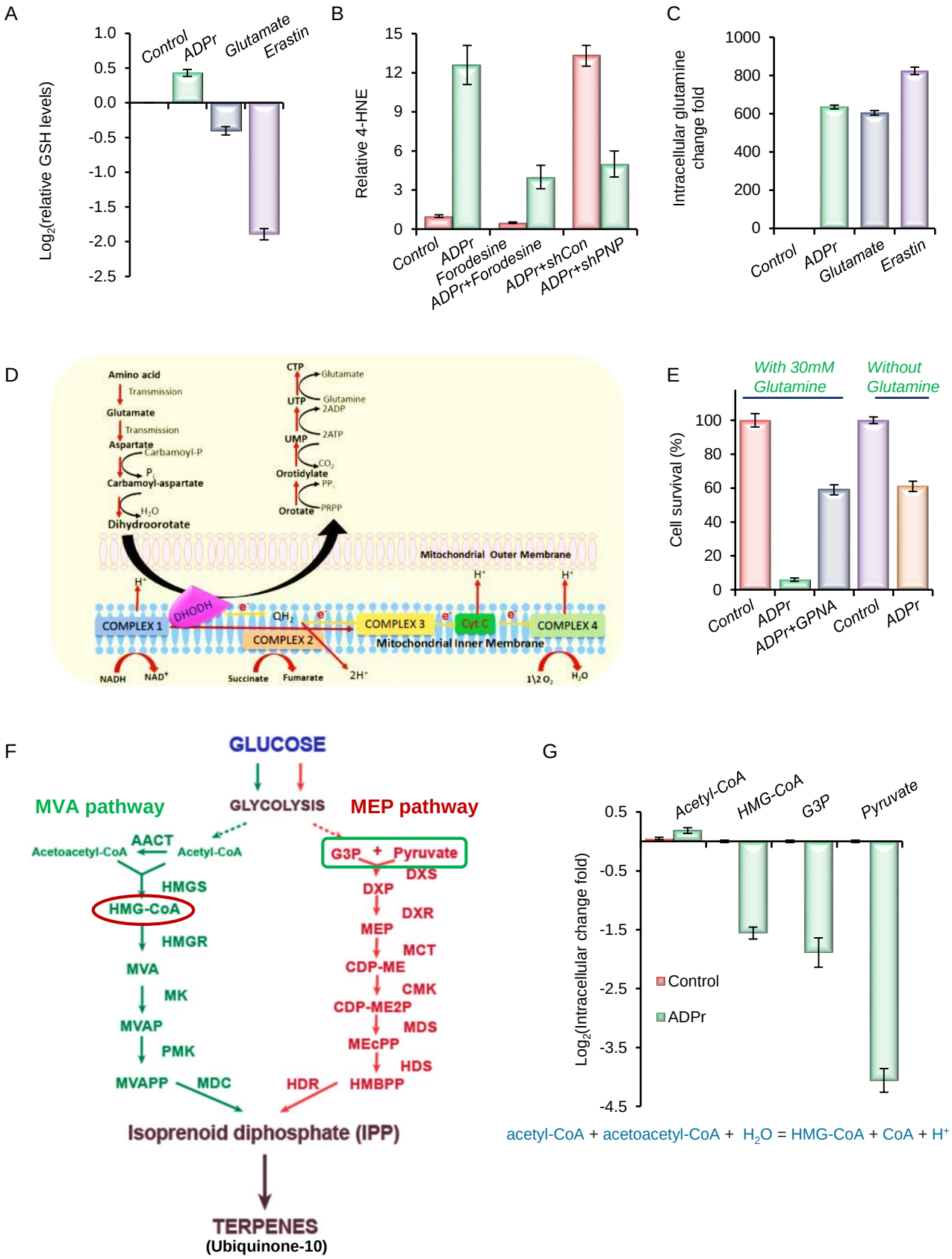

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Figure S3. ADP-ribose triggers GSH-independent ferroptosis

(A) ADP-ribose, unlike classic ferroptosis inducers (e.g. glutamate, erastin), does not depend on GSH depletion to trigger ferroptosis.

(B) Blocking the production of hypoxanthine through inhibition or depletion of PNP that catalyzes the conversion of inosine to hypoxanthine, the production of 4-HNE is significantly inhibited.

(C) The extracellular ADP-ribose stress leads to a large accumulation of glutamine in the cell.

(D) Cartoon shows the classic glutamine-dihydroorotate-quinone pathway.

(E) The lack of glutamine in the culture medium can inhibit ADP-ribose-mediated cell death.

(F) The MVA and MEP pathways.

(G) The MVA and MEP pathways were inhibited when the cell was stimulated by external ADP-ribose.

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Fig.S4 ADP-ribose induces metabolic rewiring

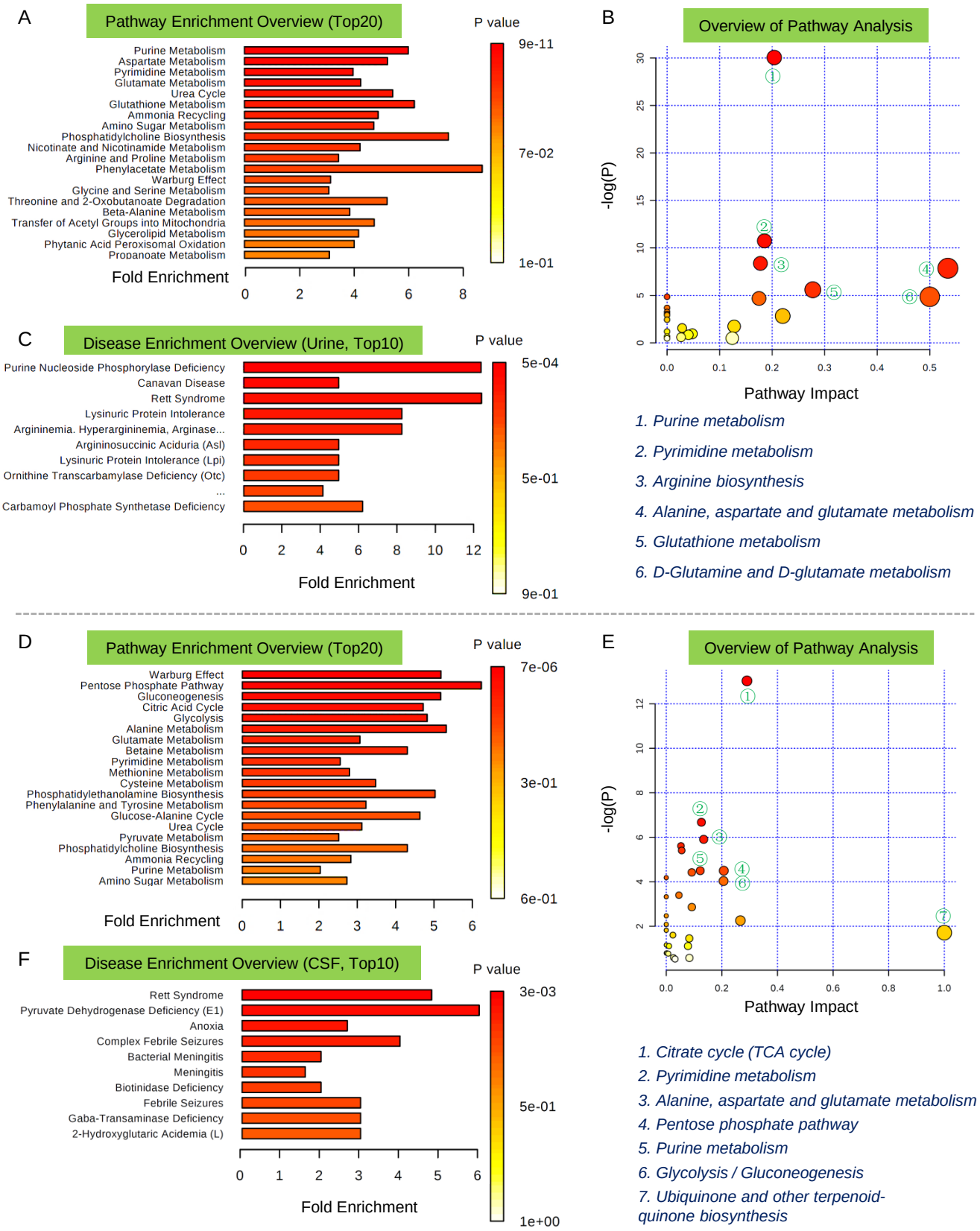

Figure S4. ADP-ribose induces metabolic rewiring

When the cell responded to the ADP-ribose in the environment, the metabolic pathways of purine, pyrimidine and glutamate were rewiring, which was similar to the effect of glutamate stress (Fig. S4, A-C). However, glutamate stress has a bias in the regulation of glutathione metabolism, while ADP-ribose stress (Fig. S4, D-F) is more biased towards the quinone biosynthesis, TCA cycle and glycolysis, which suggest that ADP-ribose-driven ferroptosis has both a mechanism similar to that of traditional inducers and its particularity.

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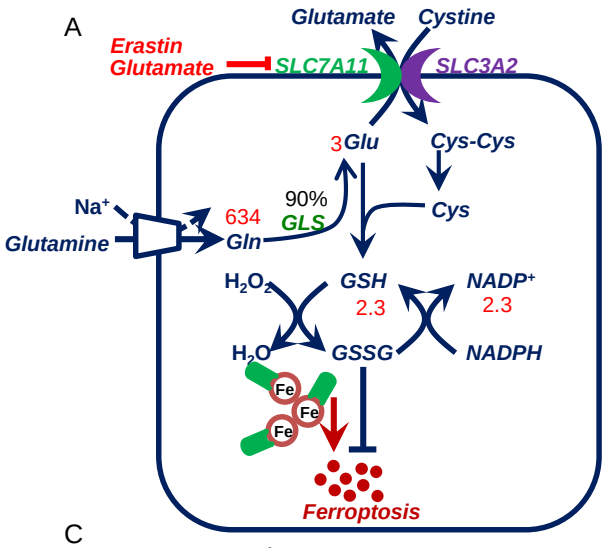
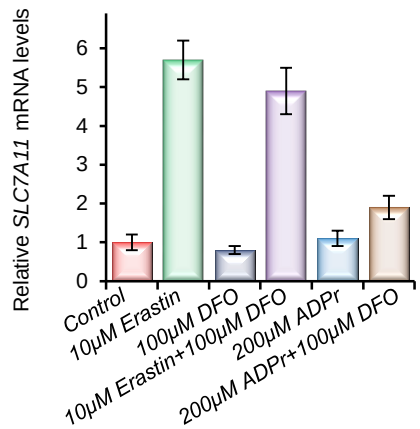
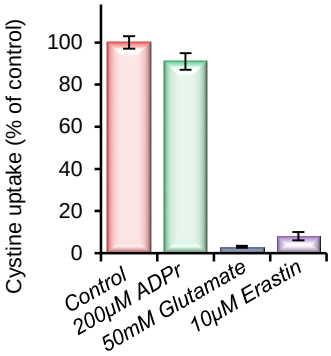
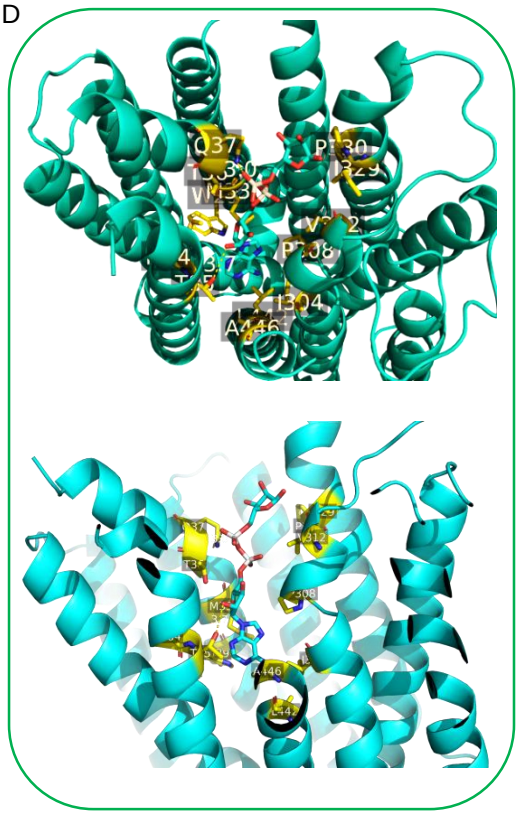
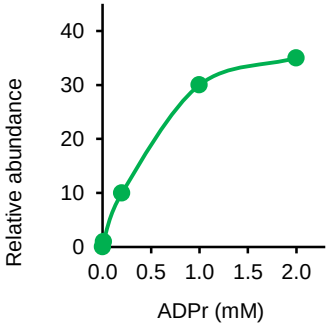
Fig.S5 ADP-ribose inhibits the Activity of ENT1

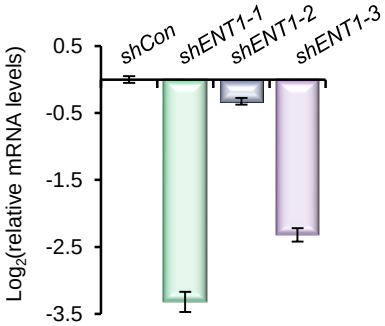


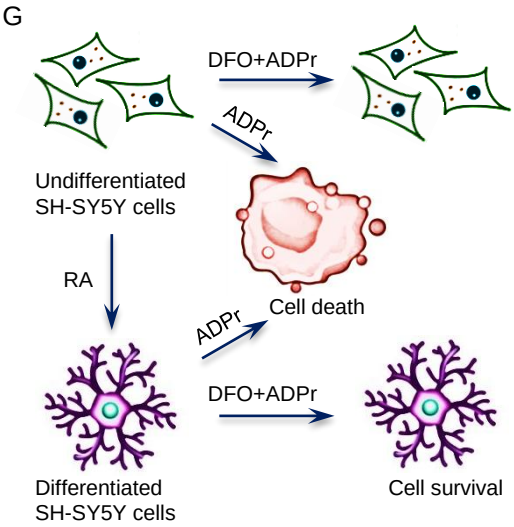












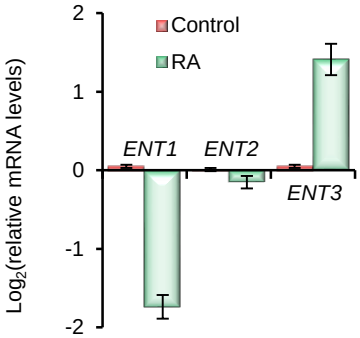


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Figure S5. ADP-ribose inhibits the activity of ENT1

(A) The classic ferroptosis pathway. System X_c^- is a disulfide-linked heterodimer composed of SLC7A11 (xCT) and SLC3A2.

(B) The substantial upregulation of SLC7A11 in SH-SY5Y cells treated with erastin. However, ADP-ribose failed to induce the upregulation of SLC7A11.

(C) Erastin (10 mM) and glutamate (50 mM) abolished the uptake of [13C]-cystine, whereas ADP-ribose had no effect on this process.

(D) The three-dimensional structural docking reconstruction of the ENT1 in complex with the ADP-ribose.

(E) A cellular thermal shift assay for evaluating influence of ADP-ribose of different concentrations on ENT1.

(F) siRNA-mediated silencing of ENT1 with three independent siRNAs.

(G) Cultures of the SH-SY5Y cell line exposed to retinoic acid (RA) to induce neuronal differentiation that demonstrated low proliferative rates and a pronounced neuronal morphology.

(H) Differentiated SH-SY5Y cells have low expression of ENT1.

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Fig.S6 ADP-ribose inhibits the Activity of NAD-dependent emzymes

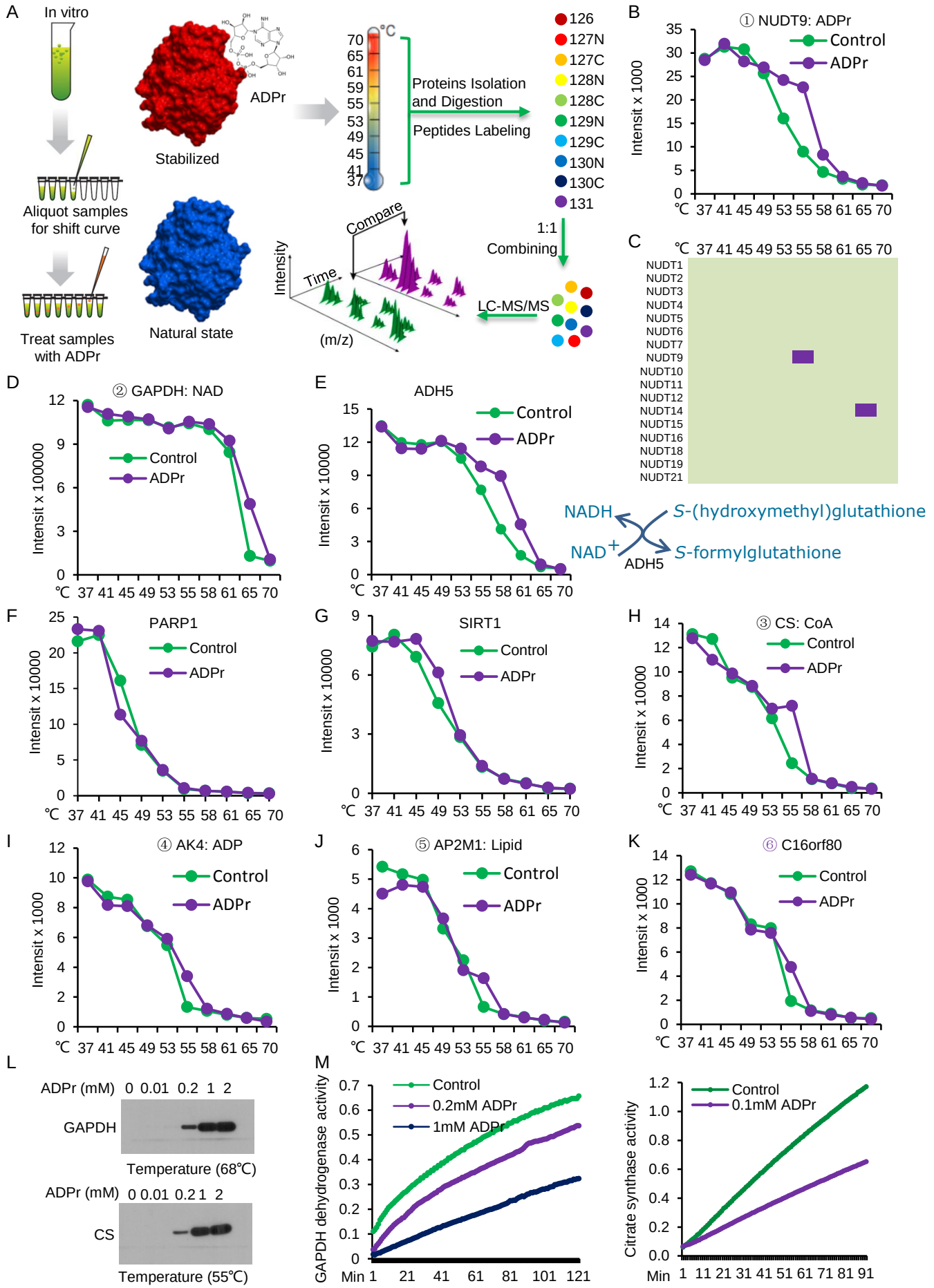

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Figure S6. ADP-ribose inhibits the activity of NAD-dependent enzymes

(A) The schematic diagram shows thermal proteome profiling (TPP) systematic screening coupled with mass spectrometry for an unbiased search of drug targets.

(B) The cellular thermal shift assay shows the specific interaction between NUDT9 and ADP-ribose.

(C) Only NUDT9 and NUDT14 of the 17 detected NUDTs have the ability to bind to ADP-ribose.

(D) The cellular thermal shift assay shows the interaction between GAPDH and ADP-ribose.

(E) The cellular thermal shift assay shows the interaction between ADH5 and ADP-ribose.

(F) The cellular thermal shift assay shows no interaction between PARP1 and ADP-ribose.

(G) The cellular thermal shift assay shows no interaction between SIRT1 and ADP-ribose.

(H) The cellular thermal shift assay shows the interaction between CS and ADP-ribose.

(I) The cellular thermal shift assay shows the interaction between AK4 and ADP-ribose.

(J) The cellular thermal shift assay shows the interaction between AP2M1 and ADP-ribose.

(K) The cellular thermal shift assay shows the interaction between C16orf80 and ADP-ribose.

(L) ADP-ribose can directly bind to GAPDH and CS.

(M) ADP-ribose can directly inhibit GAPDH and CS activities.

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Fig.S7 ADP-ribose is the therapeutic target

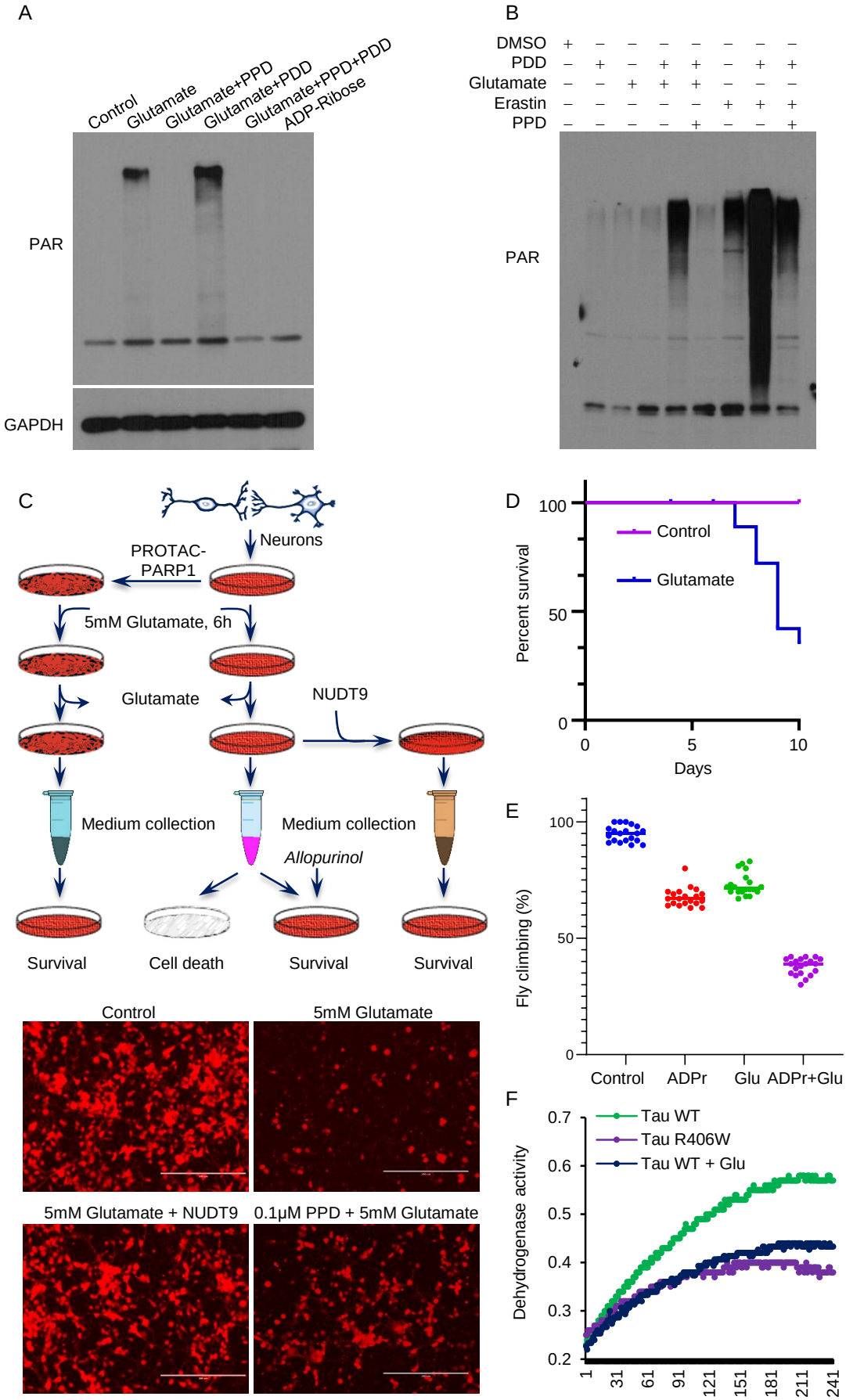

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Figure S7. ADP-ribose is the therapeutic target

(A) Glutamate can trigger PAR production in cells and PARG inhibitor, PDD, can induce PAR accumulation.

(B) PPD can effectively inhibit the production and accumulation of PAR in SH-SY5Y cells induced by glutamate treatment.

(C) The schematic diagram shows blocking the cell death induced by ADP-ribose from the three aspects.

(D) Fruit flies affected by neurodegeneration share behavioral defects and reduced lifespans, which is evidenced by the shortened lifespan of glutamate-fed fruit flies.

(E) ADP-ribose can promote climbing abnormalities caused by glutamate feeding.

(F) The dehydrogenase activity in the brain of tau^{R406W} mutant fruit flies was significantly inhibited, which is consistent with the wild-type fruit flies fed with glutamate.