Location, Location, Location: Compartmentalization of NAD+ Synthesis and Functions in Mammalian Cells

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Abstract

The numerous biological roles of NAD^+ are organized and coordinated via its compartmentalization within cells. The spatial and temporal partitioning of this intermediary metabolite is intrinsic to understanding the impact of NAD^+ on cellular signaling and metabolism. We review evidence supporting the compartmentalization of steady-state NAD^+ levels in cells, as well as how the modulation of NAD^+ synthesis dynamically regulates signaling by controlling subcellular NAD^+ concentrations. We further discuss potential benefits to the cell of compartmentalizing NAD^+, and methods for measuring subcellular NAD^+ levels.

Subcellular Compartmentalization of NAD+ Synthesis, Catabolism, and Function

Oxidized nicotinamide adenine dinucleotide (NAD^+) is a metabolic cofactor that plays essential roles in all domains of life. In over a century of research on NAD^+, great strides have been made in understanding the synthesis, degradation, and biological functions of NAD^+ (Box 1, Tables 1 and 2). Recent interest in the biology of NAD^+ has been driven by a desire to modulate its metabolic and signaling pathways to counteract illnesses stemming from metabolic disorders, tumorigenesis, inflammation, cardiovascular disease, neurodegeneration, and the onset of pathologies associated with advancing age.

NAD^+-utilizing (see Glossary) enzymes localize to specific **subcellular** compartments where they execute specific subcellular functions, leading to **compartmentalization** of NAD^+ synthesis and functions. The classic example of NAD^+ compartmentalization is the molecular distinction from its reduced counterpart, NADH, as well as from $NADP^+$ and NADPH. These differences are readily discerned in lysates and *in vitro*. Additional forms of compartmentalization, nevertheless, are not readily distinguishable in these preparations.

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These include compartmentalization between the **free metabolite** and protein-bound fractions of NAD^+, as well as the temporal and spatial partitioning of each fraction. In the cell, the abundance of NAD^+-dependent dehydrogenase enzymes leads to effective sequestration of most of the NAD^+ molecules. NAD^+ does not function as a prosthetic group and individual NAD^+ and NADH molecules are exchanged; however, the proportion of bound NAD^+ has the capacity to buffer local availability of free NAD^+.

Free intracellular NAD^+ concentrations have been measured directly at 100–120 μM in the nucleus and 50–100 μM in the cytoplasm [1–3]; variations in intracellular concentrations depend on cell type, cellular state, and growth conditions [1]. There are many NAD^+-utilizing enzymes that localize to the nucleus or cytoplasm and have $K_m(NAD^+)$ values that approximate this concentration range [4]. As such, these enzymes are poised for regulation by fluctuations in free NAD^+ concentrations. For NAD^+-consuming enzymes whose catalytic activity is regulated by signaling pathways, the availability of NAD^+ and stimulatory signals can function similarly to two-factor verification, where both are required for efficient catalytic activity of the enzyme. Thus, understanding the regulation of intracellular NAD^+ concentrations is intrinsic to understanding its impact on specific enzymatic activities *in situ*. Notably, intracellular NAD^+ is also present in mitochondria, peroxisomes, endoplasmic reticulum, and Golgi [5].

Free NAD^+ steady-state levels represent the net overall balance between local synthesis rates and consumption. Although NAD^+ molecules are derived from oxidization of NADH, this mechanism has less impact on free NAD^+ concentrations in nuclear or cytoplasmic compartments compared to synthetic pathways. This is due to the abundance of NAD^+ molecules over NADH molecules in these compartments: the free NAD^+/NADH ratio ranges between 400:1 and 700:1 in the nucleus and cytoplasm [1,2,6–9]. Thus, even if NADH levels were to change by tenfold, for example, this would only affect NAD^+ levels by 2% or less in these compartments. Mitochondrial NAD^+/NADH ratios, however, range between 5:1 and 10:1, highlighting the need to distinctly evaluate free NAD^+ in specific compartments to avoid conflation of measurements.

Non-Redundant Roles of NAD⁺ in Different Cellular Compartments

The concept that NAD^+ in different parts of the cell has non-overlapping roles is illustrated by mouse genetic models targeting individual nicotinamide mononucleotide (NMN) adenylyltransferase (NMNAT) enzymes. Many species, including humans, depend on more than one NMNAT enzyme to synthesize NAD^+ [10–15] (Table 3). Mammalian NMNAT enzymes have varying expression levels, activities, and subcellular localizations across different tissues [16–18]. NMNAT1 is nuclear, whereas NMNAT2 is associated with the Golgi and acts in the cytoplasm [19–21]. NMNAT3 can localize to the mitochondria or cytoplasm, and its localization and function depend on the cell type [19,22,23].

Distinct Subcellular Requirements for NAD⁺

Mice genetically ablated for either nuclear or cytoplasmic NMNAT activities are non-viable [24,25], indicating that the functions of the remaining enzymes cannot compensate for the specific loss of NMNAT activity in either the nucleus or cytoplasm, even if NAD^+ molecules

can traverse between these compartments. Importantly, different *Nmnat* deletions result in different underlying causes of lethality. Homozygous *Nmnat1* mice are embryonic lethal [24]. By contrast, homozygous *Nmnat2* mice are born, but are malformed and have distended bladders as well as major muscle innervation and neural development problems [25,26]. Homozygous deletion of *Nmnat3* is also lethal; the animals die postnatally from anemia owing to loss of red blood cells, reflecting a cytosolic role for the enzyme in these cells [22]. The distinct timing of when *Nmnat* knockout mice die – embryonic, perinatal, and postnatal – may additionally suggest that NMNAT activities, and possibly the requirement for various NAD$^+$ pools, are temporally regulated.

The Location of NAD$^+$ Synthesis Matters

Relocalization of NAD$^+$ synthesis to a compartment that has lost its NMNAT activity can be sufficient to rescue specific phenotypes. In cultured dorsal root ganglion neurons from rodents, loss of axonal NMNAT2 results in Wallerian axonal degeneration [27]. Restoration of as little as 25–50% of NMNAT2 activity can prevent degeneration of the axon [27]. Notably, NMNAT activity concurrently prevents the accumulation of precursor NMN that is toxic when NAD$^+$ levels are diminished, and also restores NAD$^+$ levels in these neurons [28–30]. Interestingly, overexpression of NMNAT1 in the nucleus [31] is insufficient to block induced nerve damage, but mislocalization of NMNAT1 to the axonal cytoplasm blocks degeneration [32,33]. Ectopic expression of the NMAT1 fusion protein from the *Wld^S* (slow Wallerian degeneration) mutant mouse similarly protects against induced Wallerian degeneration [34,35]. *Wld^S* generates a gain-of-function fusion protein with NMNAT activity, and its ability to block axonal degeneration depends on its localization to the axon and its catalytic activity [34,35]. These data indicate that the location of NAD$^+$ synthesis is crucial, and that NMNAT catalytic activity – not a non-enzymatic function [36] – governs this particular phenotype. Interestingly, human disease alleles of *NMNAT1* and *NMNAT2* have been identified [37–43], suggesting that subcellular NAD$^+$ regulation is similarly compartmentalized and non-redundant in humans.

Together, studies indicate that NAD$^+$ and the pathways of **NAD$^+$ biosynthesis** serve non-redundant functions in different parts of the cell, and that different pools may be pertinent at different times. The studies also indicate that regulation of NAD$^+$ synthesis can play a large role in directing the local concentrations and compartmentalization of NAD$^+$.

NAD$^+$ Compartmentalization for Regulation

NAD$^+$ compartmentalization plays an important role in regulating biological outcomes, as illustrated in the following examples.

Temporal Regulation of Circadian Rhythms

Modulation of subcellular NAD$^+$ synthesis can regulate the timing of signaling pathways. Mammalian circadian rhythms are coordinated with metabolic activity through controlled expression of NAMPT (Figure 1A) [44–47]. In both the suprachiasmatic nucleus and other circadian tissues, such as liver, NAMPT is directly upregulated by the master regulator CLOCK–BMAL1, a histone acetyl transferase and transcription factor complex [44,45].

Regulation of NAMPT, in turn, results in oscillating NAD^+ levels [44,45]. The rhythmic oscillation of NAD^+ serves as a feedback 'timer' by modulating the activities of NAD^+-dependent enzymes, including sirtuins [44–46], helping to establish the periodicity of the cycles. NAMPT oscillations also dictate mitochondrial NAD^+ levels and coordinate cellular respiration with awake periods [47]. In these cases, modulation of NAMPT levels drives the rise and fall of NAD^+ concentrations that serve to limit the duration of sirtuin activity.

Spatial Regulation by Dynamic Compartmentalization

In addition to upregulating specific biosynthetic enzymes to increase local NAD^+-dependent activity, these enzymes can be recruited or localized to cellular regions where there may be a high need. For example, NMNAT1 is recruited to genomic loci where PARP1 serves a catalytic-dependent regulatory role (e.g., gene promoters) [48,49], as well as nucleoli [50]. Likewise, NAMPT in HepG2 cells can shuttle between the nucleus and cytoplasm during the S/G2/M phases and G1 phases of the cell cycle, respectively [51].

During the early stages of **adipogenesis**, a decrease in free nuclear NAD^+ concentrations limits PARP1 activity (Figure 1B) [3]. Instead of relocating the NAD^+ biosynthetic enzymes, however, adipogenesis-inducing hormones or elevated glucose levels promote an increase in NMNAT2 protein levels, resulting in increased cytoplasmic NAD^+ concentrations. During the process, the upregulation of NMNAT2 depletes the availability of the precursor NMN, making it limiting for nuclear NMNAT1. Without sufficient NMN to robustly drive NMNAT1 activity, nuclear NAD^+ concentrations simultaneously fall. As a result, the PARP1 activity that depends on nuclear NAD^+ is limited, allowing a C/EBPβ adipogenic transcriptional program to drive differentiation of precursor cells into adipocytes [3]. Thus, increased flux of NAD^+ biosynthesis in the cytosol can also limit PARP1 activity by competing with and limiting the ability of the cell to synthesize sufficient NAD^+ in the nucleus.

Protection of Crucial NAD^+ Pools

Compartmentalization affords cells the ability to protect specific pools of NAD^+ – particularly life-crucial pools. For example, following genotoxic stress, induction of pseudohypoxic states, or chemically induced NAD^+ depletion, mitochondria retain NAD^+ levels longer than the nucleus or cytoplasm, and mitochondrial NAD^+ levels decrease only after these other compartments have been depleted (Figure 1C) [52–54]. Intriguingly, the continuous turnover of mitochondrial NAD^+ by resident consuming enzymes suggests that this pool is regularly replenished. Respiring mitochondria, in particular, depend on NAD^+-dependent SIRT3 to prevent buildup of inactivating acylation of mitochondrial enzymes [4]. The prevailing view is that minimal salvaging of NAD^+ takes place in mitochondria [53,55,56]. Depletion of the mitochondrial pool of NAD^+ only after exhaustion of the cytosolic pool hints at additional regulation between the compartments.

When NAD^+ Compartmentalization Goes Awry

Given the importance of NAD^+ compartmentalization in key biological systems such as those noted previously, non-physiological alterations in NAD^+ compartmentalization are

expected to be a driver of disease. Although direct evidence of a role for NAD^{+} compartmentalization in NAD^{+}-related pathologies (e.g., cancer, diseases of aging) is limited, recent studies are beginning to hint at its importance. For example, the same NMNAT1/ NMNAT2-driven NAD^{+} compartmentalization that regulates adipogenesis, as noted previously, is also operational in neuroblastoma cells [3]. Many questions remain to be addressed to obtain a complete picture regarding NAD^{+} compartmentalization in cancer and aging in humans.

NAD^{+} Compartmentalization in Cancer

Much of the metabolism in highly proliferative cells, including cancerous cells, centers around supporting anabolic and glycolytic pathways in the absence of mitochondrial respiration [57], suggesting that mitochondrial respiration is coordinated with the rest of the cell. Many tumors upregulate the expression of NAD^{+} biosynthetic enzymes, nicotinate phosphoribosyltransferase (NAPRT) and nicotinamide phosphoribosyltransferase (NAMPT); NAPRT is upregulated via gene amplification and NAMPT is epigenetically upregulated at its promoter [58]. These two enzymes localize predominantly to the cytoplasm and contribute to the cytoplasmic pool of NAD^{+} [59]. Increased NAD^{+} in the cytoplasm can promote cell proliferation by supporting glycolysis, as well as serving as a source for NADPH through NAD kinase (NADK). Moreover, the glycolytic enzyme GAPDH shuttles cytoplasmic NAMPT to the nucleus to increase nuclear NAD^{+} via a direct interaction promoted by cellular stress in transformed epithelial cancer lines [60].

These observations suggest the possibility that changes in subcellular NAD^{+} levels can be used to communicate between different parts of a cell when mitochondrial function is diminished or when there is a need for increased NAD^{+} outside the mitochondria. Initial rerouting of NAD^{+} from mitochondria to the cytoplasm may serve this function because cytoplasmic NAD^{+} serves as a source for the mitochondrial pool [2,55,61] and alterations in its distribution can affect proliferation [61]. As such, reorganization of subcellular NAD^{+} levels could represent an early initiating step in tumorigenesis. Because many tumor types broadly depend on upregulated NAD^{+} for proliferation [58], targeting this pool of NAD^{+} may have potential as a therapeutic intervention.

NAD^{+} Compartmentalization in Aging

Declining NAD^{+} levels have been proposed to underlie the onset of pathologies associated with advanced age, and link age as a risk factor to diseases such as neurodegeneration and cancer. As suggested by preclinical data, lowered NAD^{+} levels diminish the ability of cells to replenish niches, counteract inflammation and senescence, repair damage, and regulate metabolic pathways [62]. Moreover, lowered NAD^{+} levels can directly contribute to cellular stress by promoting a reductive environment. Nevertheless, the extent to which decreased NAD^{+} levels contribute to aging in healthy humans, notably, is still debated [63–67]. Despite evidence for age-dependent decreases of NAD^{+} in preclinical models, these observations have not always translated directly to humans. Age-related reductions in NAD^{+} in humans may require particular stressors, be limited to specific cell types or subcellular compartments, or represent modulations of specific pathways. Moreover, alterations in NAD^{+} compartmentalization generally, or alterations in NAD^{+} levels in a specific compartment,

in the pathologies of aging have not been determined. As such, the complete picture regarding specific cell types, subcellular compartments, and the extent of NAD^+ loss in human aging and disease models remains elusive.

On the Outside Looking In: An Extracellular Compartment for NAD⁺?

In this review we have focused on the synthesis and functions of NAD^+ in intracellular compartments. NAD^+, however, is also present in the extracellular milieu as extracellular NAD^+ ($eNAD^+$) [68], which may be considered as another compartment for NAD^+.

Origins and Consumption of eNAD⁺

Despite of the presence of dimerized extracellular NAMPT (eNAMPT), which can make NMN [69], the enzymes necessary to catalyze the final step of NAD^+ synthesis are not known to reside extracellularly [70]. This has raised questions about the provenance of $eNAD^+$. Current thinking is that $eNAD^+$ arises from NAD^+ that is synthesized intracellularly and either released upon cell lysis or through pore-forming proteins, such as connexin 43 (Cx43) hemichannels [68,71,72].

More is known about the consumption of $eNAD^+$ than about its origins. The structurally and functionally related NAD^+ glycohydrolases CD38 and CD157 are ectoenzymes that hydrolyze $eNAD^+$ to cyclic ADP-ribose (cADPR) and ADPR, which in turn can serve as paracrine second messengers to ultimately mediate intracellular calcium mobilization and signaling [73–75]. Notably, CD38 also targets circulating NAD^+ precursors, such as NMN and nicotinamide riboside (NR), for degradation, and the liberated products can be directed to NAD^+ biosynthetic pathways inside the cell [76,77]. Studies in *Cd38* knockout mice have demonstrated that CD38 is a major consumer of NAD^+ in mammals. Thus, CD38 has the potential to govern the biology of $eNAD^+$ by controlling its levels [78].

Biology of eNAD⁺

Much of our understanding of the biology of $eNAD^+$ comes from studies using *Cd38* knockout mice, which have revealed a variety of outcomes that link CD38 to physiological functions, presumably through its ability to degrade $eNAD^+$ and generate cADPR and ADPR as second messengers [73,74]. Defects observed in the knockout mice include impaired neutrophil chemotaxis, defective oxytocin release, and aberrant social behavior, as well as altered tumorigenic potential [73,79,80]. Several recent studies have reported that both intracellular and extracellular NAD^+ levels decline with age [81], which occurs with a concurrent increase in CD38 protein levels in various tissues [78,82]. *Cd38* knockout mice have twice the levels of NAD^+ and are resistant to the age-associated decline in NAD^+ [83,84]. By contrast, mice engineered to overexpress CD38 have lower levels of NAD^+ and exhibit age-related mitochondrial dysfunction mediated, in part, by regulation of SIRT3 activity [78]. Interestingly, adipose tissue-specific overexpression of NAMPT in mice, which maintains plasma eNAMPT levels and NAD^+ synthesis in various tissues, is associated with improved glucose-stimulated insulin secretion, photoreceptor function, and cognitive function [85]. Although the pathway that would lead from NMN synthesis by eNAMPT to

increases in eNAD$^+$ is unclear, the parallels between *Cd38* knockout and NAMPT overexpression in mice suggest a shared mechanism involving eNAD$^+$.

Adding more complexity, a cell-surface transporter, SLC12A8, was recently reported to mediate the influx of extracellular NMN in the gut [86]. In addition, intracellular nicotinamide riboside kinase (NMRK) has been reported to be rate-limiting for extracellular NMN and NR in NAD$^+$ synthesis [87]. These paradoxical mechanisms, where extracellular NMN is either directly imported or requires dephosphorylation to enter cells as NR, may very well represent mechanisms of compartmentalization *in vivo* that call for further study. Overall, the available evidence suggests that, although the extracellular space does not function as a separate compartment for NAD$^+$ synthesis, it may represent a separate compartment for NAD$^+$ function. As such, it is likely to be subject to the same temporal and spatial considerations discussed previously for intracellular NAD$^+$.

Methods to Study NAD$^+$ Compartmentalization

The interior of any cell is not a uniform milieu: cells are organized with membrane-bound organelles, macromolecular scaffolding and tracks, and subcompartments with distinct viscosities. The complexities of NAD$^+$ compartmentalization – including being able to distinguish free intracellular fractions from total NAD$^+$ levels – present challenges for monitoring and determining NAD$^+$ concentrations in real time and *in vivo*. An ideal method would dynamically monitor free NAD$^+$ in an organism with subcellular resolution, be minimally invasive, not compromise the existing pool, and be able to overlook circumstantial differences in oxygen levels and pH in its report of free NAD$^+$. Although each currently available method has its trade-offs, and an all-encompassing perfect solution remains elusive, much progress has been made towards addressing the intricacies of monitoring intracellular NAD$^+$.

Trade-Offs in Spatial and Temporal Resolution

Many commonly applied techniques to monitor NAD$^+$ rely on cellular extracts. Unfortunately, the preparation of extracts compromises intrinsic spatial information from the sample, especially at the subcellular scale, and cannot be used reliably to evaluate free NAD$^+$. These are limitations of colorimetric assays, chromatography, nuclear magnetic resonance, and mass spectrometry (MS). Methods to isolate organelles or subcellular fractions cannot ensure that metabolites remain in their original subcellular compartment (Figure 2). *In situ* adaptations of these techniques (e.g., MS imaging and gold nanocluster-based probes) are still subject to the challenges of precisely separating out and quantifying free NAD$^+$ from the more abundant protein-bound NAD$^+$ fraction. Available determinations and estimations indicate that the free fraction only comprises 12–25% of total NAD$^+$ [1,2,6]; total NAD$^+$ measurements range from ~300 μM [52,88,89] to as high as 800 μM [90,91] in mammalian cells. The higher abundance of the protein-bound fraction likely overwhelms the detection of free NAD$^+$ in total measurements. In addition, extract-based approaches cannot repeatedly measure from the same cell over time. A minimally invasive *in vivo* magnetic resonance NAD$^+$ assay has been developed that can distinguish NAD$^+$ from NADH and has been used in physiological settings for patient diagnosis [89,92]. The major trade-offs,

however, are that it cannot distinguish free from bound fractions, and the approach is limited for observing fluctuations at the subcellular scale.

Pitfalls of Indirect Estimations

Indirect projections based on lactate dehydrogenase activity or NADH have historically been used successfully to estimate steady-state levels during homeostasis [6,7]. However, the assumptions necessary to calculate these concentrations are not always valid in all scenarios [8,93]. In particular, observed changes in NADH do not necessarily reflect alterations in free NAD$^+$ levels, and monitoring changes in free NADH levels can overestimate resulting changes in NAD$^+$ concentrations. This is due to the disproportionately high NAD$^+$/NADH ratio in many compartments.

Genetically Encoded Approaches

NAD$^+$, unlike NADH, has no intrinsic fluorescence, and absorbance cannot distinguish its free fraction [7,94]. Currently, the best methods for measuring compartmentalized NAD$^+$ have used genetically encoded sensors (Figure 2) [1,2]. This technique permits full control over the expression of the probe, including restricting its expression to specific cell types, temporally controlling when it is expressed or active, and targeting it to subcellular localization via encoded localization sequences or tethering [95]. In addition, genetically encoded sensors are readily adaptable to *in vivo* applications [95,96].

An early NAD$^+$ sensor, PARAPLAY, uses the catalytic domain of PARP1. This domain can be auto-modified by poly(ADP-ribose) in an NAD$^+$-dependent manner, and the extent of modification as assessed by Western blotting depends on NAD$^+$ availability [5,56]. Appreciable challenges, nevertheless, include applying this approach to quantitatively measure NAD$^+$ in cells and ensuring that expression of the catalytically active probe minimally affects the local NAD$^+$ pool and its downstream targets. Multiple genetically encoded ratiometric NADH/NAD$^+$ sensors also have been developed and have been invaluable for NADH intracellular measurements [8,9,97,98]. However, because the concentration of free NAD$^+$ exceeds that of free NADH, the current ratiometric NADH/NAD$^+$ sensors become saturated and cannot be used in cells for NAD$^+$ measurements.

To address the need for dedicated NAD$^+$ sensors, two first-generation *in situ* sensors were developed [1,2]. A semisynthetic sensor called NAD$^+$-Snifit was developed by the laboratory of Johnsson based on their innovative Snifit (snap-tag indicator fluorescent intramolecular tether) system [1,99]. Snifit designs work by an analyte-dependent conformational change to the probe that alters fluorescence resonance energy transfer (FRET). In the case of NAD$^+$-Snifit, binding of free NAD$^+$ induces an intramolecular interaction to increase FRET between a donor associated with a tethered SNAP-tag and an energy acceptor associated with a Halo-tag directly adjacent to the NAD$^+$ binding protein (sulfapyridine). The modularity of the Snifit design permits its successful adaptation for the detection of ions, neurotransmitters, drugs, and other metabolites, including NADP$^+$ and NADPH [100]. Moreover, Snifits can use fluorescent proteins, synthetic dyes, or luciferase to provide resonant energy; readouts can be either fluorescence or bioluminescence. A series

of sensitive bioluminescent Snifit sensors for NAD$^+$ – headlined by NAD-cpLuc and NAD-cpLuc2 – were also recently developed as *in vitro* paper-based assays to facilitate point-of-care readings from biological samples and extracts [101]. These new sensors cover NAD$^+$ concentrations from 10^{-8} to 10^{-4} M using a color-change readout of emitted light from blue to red upon NAD$^+$ detection. They represent the most sensitive sensors to date and have multifold dynamic ranges. Although not technically intended for use *in situ*, they hold great promise for measurements of eNAD$^+$ that may be at much lower concentrations relative to intracellular NAD$^+$.

An independent NAD$^+$ sensor was initiated as a collaboration between the laboratories of Goodman and Cohen [2], with the goal of ultimately facilitating *in vivo* studies of intracellular NAD$^+$. The fluorescence readout of this sensor is fully genetically encoded and the complete sensor is encoded by a single polypeptide. The sensor combines a permutated NAD$^+$-binding domain modeled after *Enterococcus faecalis* LigA with the circularly permutated fluorescent protein cpVenus$^{144/145}$. Binding of local free NAD$^+$ is detected by decreased fluorescence intensity from cpVenus. As a general strategy, circularly permutated fluorescent proteins have aided intracellular sensor designs since the late 1990s, notably the original development of a genetically encoded calcium indicator [102,103].

The development of these sensors has allowed measurement of free subcellular NAD$^+$ for the first time [2]. By achieving subcellular resolution, the sensors established that (i) intracellular NAD$^+$ concentrations are compartmentalized, (ii) subcellular regulation differs by cell type, (iii) local free NAD$^+$ levels match the reported K_m(NAD$^+$) values of many NAD$^+$ signaling enzymes, and (iv) lowering NAD$^+$ levels limits the activity of these enzymes in cells [1,2]. Moreover, the new methodologies represent founding tools that can be customized and optimized for the pursuit of new research directions.

Significant technical challenges remain for moving the technologies *in vivo* and expanding their utility to different physiological compartments. Better evaluation of long-term tolerance, minimizing buffering effects from expression of the probe, and increasing the dynamic range of readouts would all represent important improvements. Advances continue to be made; the developers of SoNAR, originally an NADH/NAD$^+$ sensor, recently re-engineered it as FiNAD to preferentially monitor relative NAD$^+$ levels [104]. The advantages of FiNAD include a large dynamic range (like its predecessor); utility for measurements in bacteria, vertebrates, mammalian cells, and primary human cells; and (like NAD$^+$-Snifit) utility in experiments using high levels of precursor supplementation. However, FiNAD has a K_m(NAD$^+$) of ~1.3 mM, limiting its use in mammalian cells. With a K_m(NADH) of ~100 μM, crossreactivity with NADH greater than 10 μM further limits the use of FiNAD for mitochondria measurements, as well as in anaerobic or hypoxic cultures [104].

A Call for Additional Designs

Understanding the advantages and limitations of each technology is important. In lieu of a single perfect method for monitoring intracellular NAD$^+$ across all species, compartments, and scenarios, the future will likely include multiple sensors where each serves a specialized purpose. As such, second-generation *in situ* technologies will need to be developed, and will

likely require combinations of both rationale design and unbiased screening [103]. One can imagine a molecular toolbox of customized NAD$^+$ sensors for specific queries. For example, the major differences between fluorescent protein- and NAD$^+$-Snifit-based designs represent distinct advantages and limitations depending on the circumstances. The current fluorescent protein-based sensors are ultimately sensitive to pH and oxygen levels owing to the cpYFP or cpVenus component [2,104]. NAD$^+$-Snifit can utilize pH and oxygen-insensitive FRET pair dyes [1], but reliance on two exogenously applied dyes may spectrally limit options for **multiplexing** NAD$^+$-Snifit *in vivo*. In this regard, the utility of each sensor should be evaluated based on its intended application. For example, a sensor designed for measurements in mammalian cells does not need to respond robustly to changes in *E. coli* [104].

Additional utility will come from different sensors that are sensitive for NAD$^+$ at different ranges, reflecting differences in the expected concentrations in various cellular milieus, cell types, and species. Intracellular measurements for peroxisomes, endoplasmic reticulum, and Golgi, are currently lacking, and the possibility of focal regulation of NAD$^+$ at specific chromatin loci or sites of DNA damage has not been addressed. In addition, expanded palettes for sensors with new wavelengths would be welcomed, especially in the IR range to minimize **phototoxicity**. Lastly, multiplexing these sensors with detection of NADH, NADP$^+$, and NADPH molecules and the complement of metabolomic data will be crucial to uncover the full role of compartmentalized intracellular NAD$^+$ signaling.

Concluding Remarks

NAD$^+$ is a crucial metabolite and signaling molecule whose detailed functions and biology are incompletely characterized, at best. We are only beginning to elucidate how the temporal and spatial compartmentalization of NAD$^+$ contributes to its numerous biological roles. The emerging view is that NAD$^+$ concentrations are partitioned and dynamically modulated by NAD$^+$ synthesis within the cell. Determining the extent of these mechanisms *in vivo* and their impact on signaling pathways will be a rich area for future research (see Outstanding Questions). Continued development of new methodologies to measure intracellular NAD$^+$ will be essential to this effort. Because NAD$^+$ is crucial for how organisms in all domains of life organize and extract energy, NAD$^+$ regulation is integral to every discipline and field of study in biology and medicine. Not only are its chemistry and mechanisms part of the history of biochemistry, but its broad biological impact will undoubtedly also continue to bring together expertise across multiple disciplines in the future. The next wave of discoveries in NAD$^+$ biology will be driven by collaborations among scientists with distinct skillsets, including physiologists, enzymologists, cell and molecular biologists, evolutionary biologists, engineers, chemists, physicists, and bioinformaticians.

Acknowledgments

Glossary

Adipogenesis	the developmental process by which precursor cells develop into adipocytes (fat cells)
Compartmentalization	separated into isolated or distinct subcellular locales
Ectoenzyme	an enzyme that is located on, and has catalytic activity at, the surface of a cell, directed towards the exterior of the cell
Free metabolite	the fraction of a metabolite that is not currently bound to or associated with protein. Typically, this can be considered as the amount of available metabolite
Multiplexing	combining more than one assay in a simultaneous analysis
NAD$^+$ biosynthesis	the ordered and regulated series of chemical reactions that lead to the generation of an NAD$^+$ molecule
NAD$^+$ utilization	the use of NAD$^+$ either as a coenzyme in metabolic reactions or as a substrate for degradation
Phototoxicity	toxic effects on the cell resulting from exposure to illumination from lasers and high-intensity arc-discharge lamps
Salvage pathway	a biosynthetic pathway that recycles intermediates from a degradative process
Subcellular	refers to a defined location, organelle, or structure within a cell. It can also refer to an event or molecule that occurs or resides within the confines of a particular space within a cell

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Box 1.

NAD$^+$ Synthesis and Catabolism

NAD$^+$ comprises two nucleotides (Figure IA). The steady-state levels of NAD$^+$ are determined by the balance between synthesis and utilization, as described subsequently.

NAD$^+$ Synthesis

The biosynthesis of NAD$^+$ occurs in multiple pathways, including *de novo* synthesis and **salvage pathways** [4,105] (Table 1 and Figure IB). *De novo* synthesis originates with tryptophan, and, through the kynurenine pathway, converges with dedicated NAD$^+$ synthesis pathways through quinolinic acid (QA). Quinolinate phosphoribosyltransferase (QPRT) catalyzes the formation of nicotinic acid mononucleotide (NAMN) from QA, which is subsequently converted to nicotinic acid adenine dinucleotide (NAAD) by one of the three NMNAT enzymes. The final step is the amidation of NAAD by *NAD synthetase* (NADS), leading to the production of NAD$^+$ (Figure IB) [4,105]. *De novo* synthesis is generally limited to the liver, kidney, and some immune cells [91,106].

In the mammalian salvage pathways, NAD$^+$ is synthesized from the dietary precursors nicotinic acid (NA), NAM, or NR. In the NA ('Preiss–Handler') salvage pathway, NAMN is produced from NA by NAPRT and is ultimately converted to NAD$^+$ by NADS (Figure IB). In the NAM and NR pathways, NMN is produced from NAM by NAMPT or from NR by NMRK1, respectively. Although most tissues heavily rely on NMN and NR pathways, there is variability *in vivo* for the tissue types that can synthesize NAD$^+$ through the Preiss–Handler pathway [91]. Nonetheless, the final step in both pathways is the synthesis of NAD$^+$ from NMN and ATP by one of the NMNAT enzymes (Figure IB) [4,105].

NAD$^+$ Utilization

NAD$^+$ is a cofactor in cellular redox reactions in key metabolic pathways, where it is reduced to NADH without being catabolized [107]. In other contexts, however, NAD$^+$ is consumed (Table 2). For example, PARP enzymes hydrolyze NAD$^+$ to release ADPR groups that are used for the covalent mono- or poly(ADP-ribosyl)ation of proteins [108,109], DNA [110–113], and RNA [114,115]. Likewise, some sirtuins also catalyze mono(ADP-ribosyl)ation of proteins, whereas others catalyze deacylation using ADPR as an acceptor of acyl groups removed from covalently modified proteins [116,117]. NAD$^+$-degrading enzymes include vertebrate NAD$^+$ glycohydrolases, CD38 [76,77], and SARM1 [30,122] (Table 2). These NADases, which are enzymes that catalyze the hydrolysis of NAD$^+$ to ADP-ribose and nicotinamide, have been identified in bacteria, fungi, and mammals, and some are membrane-anchored [118–121]. NAD$^+$ is also phosphorylated by NAD$^+$ kinase to generate NADP$^+$. NADP$^+$ is further reduced by dehydrogenases to generate NADPH, an important cofactor in biosynthetic pathways and for neutralizing reactive oxygen species generated by metabolic activity [123]. Finally, NAD$^+$ is used for the 5$'$ capping of mRNAs, which promotes metabolite-directed mRNA turnover in mammals [124].

Highlights

NAD^+ serves an essential role as an electron acceptor (via hydride transfer) in central carbon metabolism. In the absence of intracellular NAD^+, cells cannot produce ATP. However, even moderate diminishments in NAD^+ levels can limit the signaling activity of NAD^+-consuming enzymes.

NAD^+ concentrations differ in different parts of the cell, and there are distinct subcellular requirements for NAD^+.

Dynamic modulation of subcellular, and possibly extracellular, NAD^+ concentrations represent an emerging mechanism for regulating specific NAD^+-dependent pathways.

Compartmentalization of NAD^+ helps to time responses, communicate cellular status, and protect crucial NAD^+ pools.

Genetically encoded sensors represent promising approaches for additional development to generate a molecular toolbox for measuring and studying fluctuations in levels of compartmentalized NAD^+.

Outstanding Questions

What are the regulatory roles for the compartmentalization of NAD$^+$ *in vivo*? For a full understanding, questions will need to be addressed in cells, as well as at the organ and organismal levels, in both physiological and disease contexts. The application of genetically encoded sensors in model organisms will be crucial for achieving these goals.

How are the roles for NAD$^+$ compartmentalization conserved in different organisms? Different species possess variations in their NAD$^+$ biosynthetic pathways.

How do NAD$^+$ levels in specific compartments interplay with NADH, NADP$^+$, and NADPH levels, as well as with other signaling metabolites?

What is the variation of compartmentalized NAD+ in individual cells?

How do fluctuations in the proportion of protein-bound NAD$^+$ affect free NAD$^+$ concentrations in different parts of the cell?

Can we design methods to precisely regulate NAD+ concentrations in specific cellular compartments?
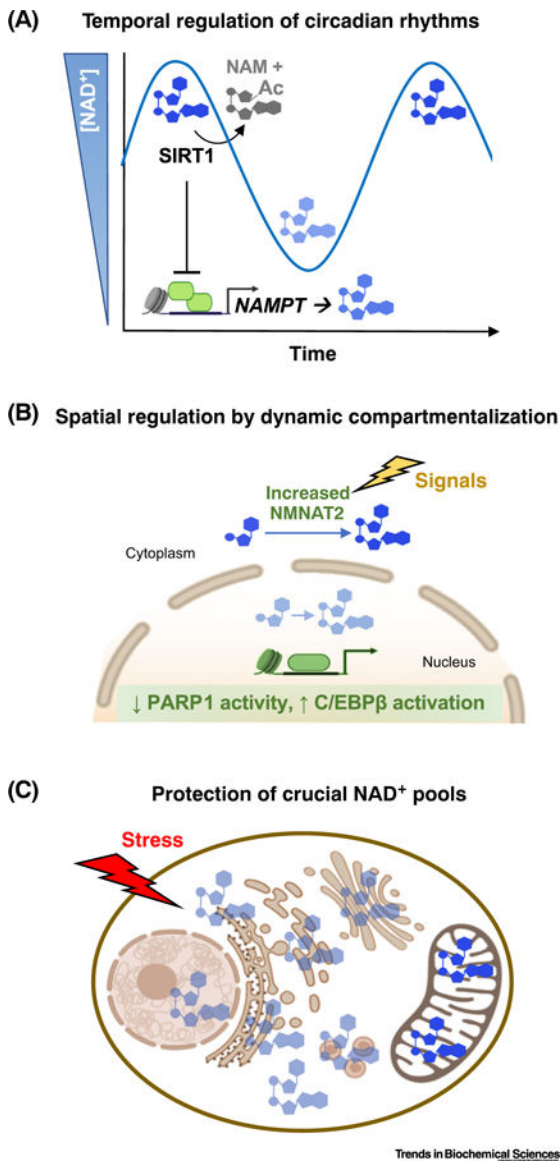
Figure 1. NAD$^+$ Compartmentalization for Regulation.

The levels of free NAD$^+$ are tightly controlled both temporally and spatially to precisely modulate signaling in cells. (A) Temporal fluctuations in NAD$^+$ concentrations regulate circadian periodicity rhythms. (B) Spatial partitioning of NAD$^+$ biosynthetic pathways permits signal-dependent initiation of adipogenic differentiation. (C) NAD$^+$ compartmentalization may protect and prioritize support for particularly crucial cellular processes (e.g., in the mitochondria) in response to stress when other subcellular stores are depleted. Abbreviation: Ac, acylation.

A

B
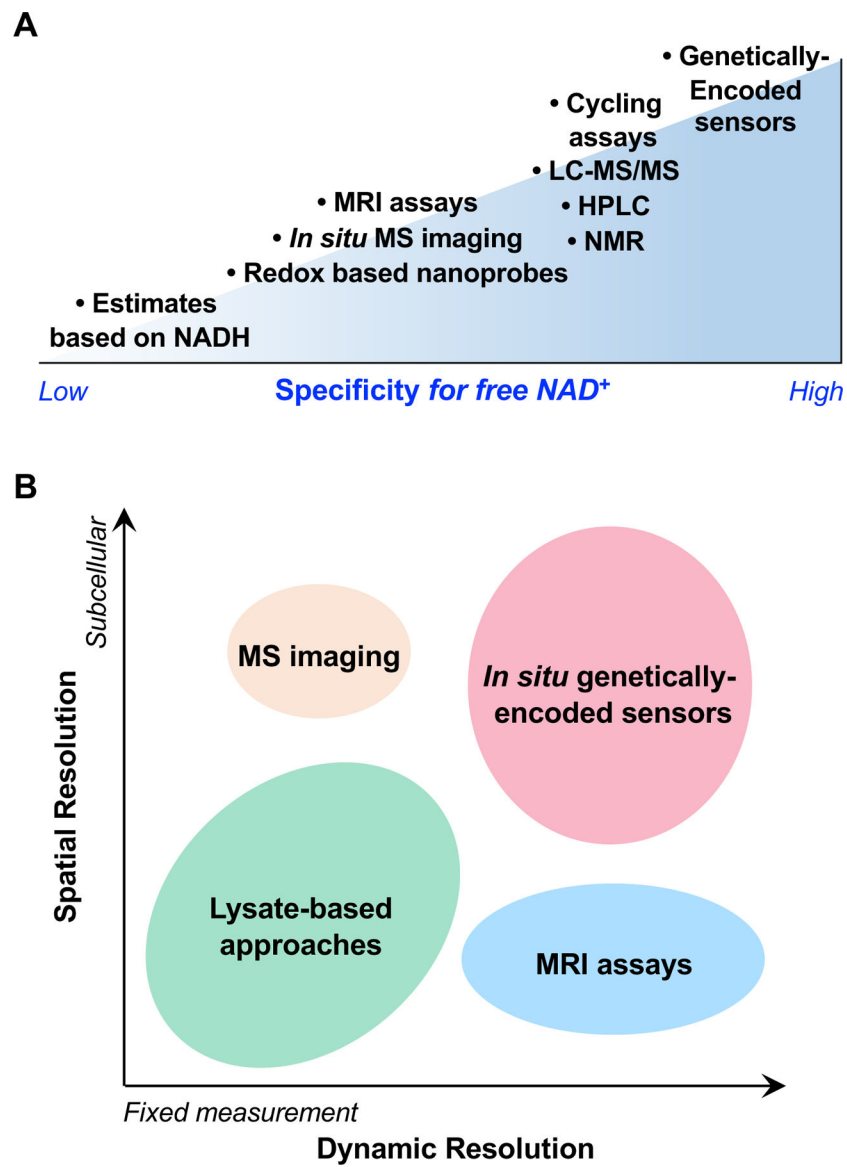
Figure 2. Specificity and Resolution when Measuring Intracellular NAD$^+$.
Schematic representations of the specificity and resolution of various methods for measuring intracellular NAD$^+$. (A) Methods are broadly ordered by their ability to both directly and specifically measure the free fraction of intracellular NAD$^+$. Consideration of the entire methodology is taken into account. For example, analytical methods, such as LC-MS/MS, HPLC, and NMR, directly and very specifically measure intracellular NAD$^+$; however, the preparation of biological samples for analysis may conflate free and bound fractions. (B) Methods are organized according to their ability to obtain dynamic measurements from the same locale, as well as by their spatial resolution. Abbreviations: LC, liquid chromatography; MRI, magnetic resonance imaging; MS, mass spectrometry; NMR, nuclear magnetic resonance.

A

B
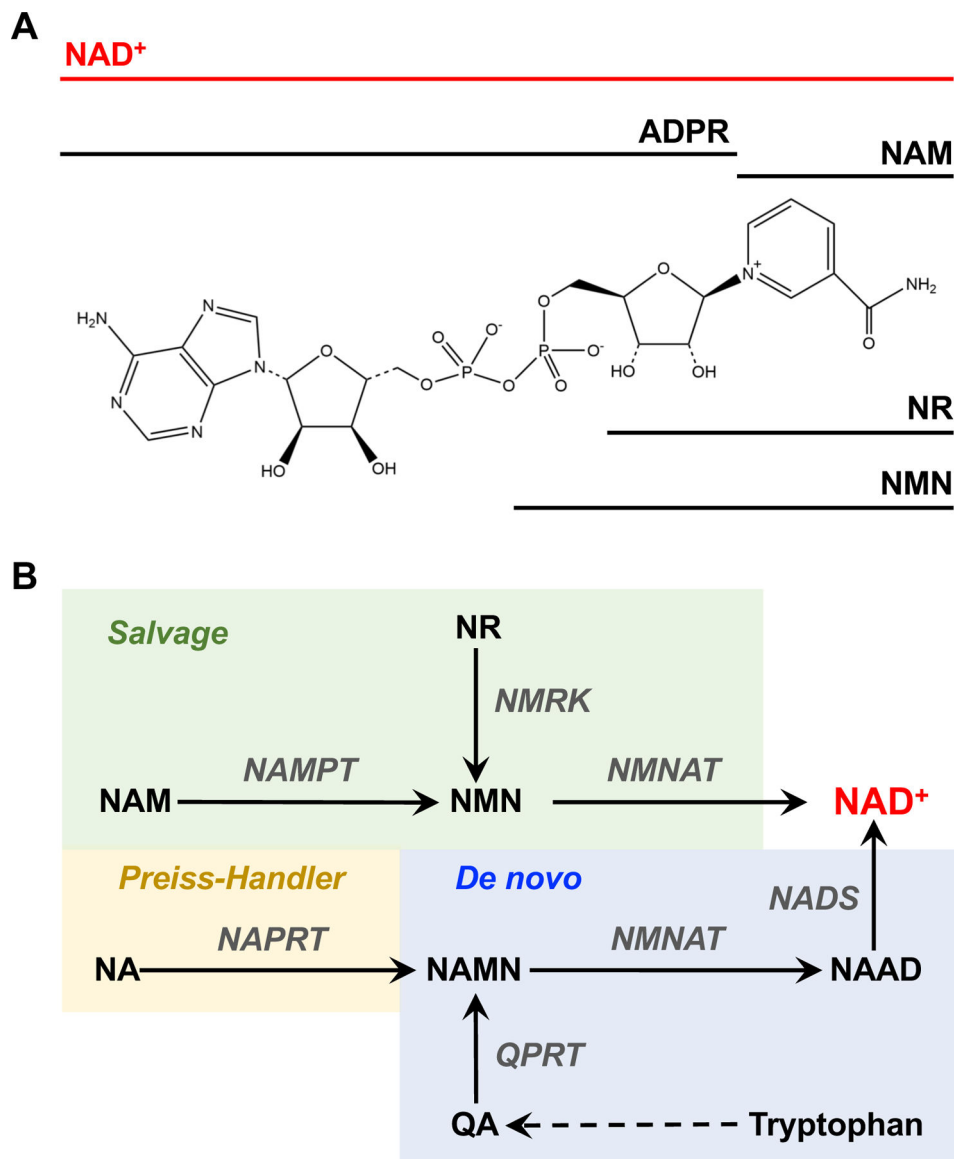
Figure I. Chemical Structure and Biosynthesis of NAD+.
(A) Chemical structure of oxidized NAD+. NAD+ comprises two nucleotides, one containing an adenine nucleobase and the other containing nicotinamide, joined through their phosphate groups. The location of various chemical moieties contained within NAD+ are indicated: ADP-ribose (ADPR), nicotinamide (NAM), nicotinamide riboside (NR), and nicotinamide mononucleotide (NMN). (B) NAD+ is synthesized *de novo* in a pathway leading from tryptophan (blue), as well as in 'salvage' pathways: (i) the nicotinic acid (NA) ('Preiss–Handler') salvage pathway (yellow), (ii) the nicotinamide mononucleotide (NMN) salvage pathway (green), and (iii) the nicotinamide riboside (NR) salvage pathway (green). The abbreviations for the enzymes (gray text) and the intermediates (black plain text) are defined in Table 1.

Table 1.

Key Enzymes in the NAD$^+$ Biosynthetic Pathways

Enzyme	Activity	Pathway(s)
Quinolinate phosphoribosyl transferase (QPRT)	Catalyzes the formation of nicotinic acid mononucleotide (NAMN) from quinolinic acid (QA)^a	*De novo* pathway
NAD$^+$ synthetase (NADS)	Catalyzes the amidation of NAAD, leading to the production of NAD$^+$	*De novo* pathway
Nicotinic acid phosphoribosyl transferase (NAPRT)	Catalyzes the formation of NAMN from nicotinic acid (NA); rate-limiting step [125]	NA ('Preiss–Handler') salvage pathway
Nicotinamide phosphoribosyl transferase (NAMPT)	Catalyzes the production of nicotinamide mononucleotide (NMN) from NA; rate-limiting step [126]	NMN salvage pathway
Nicotinamide riboside kinase (NMRK)	Catalyzes the production of NMN from NR; rate-limiting step [87,127]	NR pathway
Nicotinamide mononucleotide adenylyltransferase (NMNAT)	Catalyzes the formation of nicotinic acid adenine dinucleotide (NAAD) from NAMN, or of NAD$^+$ from NMN	*De novo* and salvage pathways

^{a}The *de novo* NAD$^+$ synthesis pathway originates with tryptophan, and through the kynurenine (Kyn) pathway converges with dedicated NAD$^+$ synthesis pathways via quinolinic acid (QA). The Kyn pathway also feeds other metabolic pathways. Indoleamine-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO) catalyze the first and rate-limiting step of the Kyn pathway. QPRT is positioned to catalyze the rate-limiting step of the specific *de novo* NAD$^+$ biosynthesis portion of the pathway, but whether it does so is debated in the literature and may be context-specific [125,128–130].

Table 2.

Major Enzymes That Use or Consume NAD$^+$

Enzyme	Activities
Poly(ADP-ribose) polymerases (PARPs)	Cleave NAD$^+$ to release ADP-ribose (ADPR) groups that are used for the covalent mono- or poly(ADP-ribosyl)ation of proteins, DNA, and RNA
Sirtuins	Some sirtuins (e.g., SIRT1) catalyze deacylation of proteins using ADPR as an acceptor of acyl groups (i.e., acetyl, succinyl, malonyl, etc.) Other sirtuins (e.g., SIRT4 and SIRT6) catalyze mono(ADP-ribosyl)ation of proteins
CD38	Ectoenzyme that has both NAD$^+$ glycohydrolase and ADP-ribosyl cyclase activities, which may function in decreasing extracellular NAD$^+$ levels
SARM1	Has both NAD$^+$ glycohydrolase and ADP-ribosyl cyclase activities
NAD$^+$ kinase	Phosphorylates NAD$^+$ to generate NADP$^+$; this diminishes the pool of NAD$^+$ available for NAD$^+$-specific enzymes and processes

Table 3.

Homologs of Nicotinamide Mononucleotide Adenylyltransferase[a]

Species	NMNAT1	NMNAT2	NMNAT3
H. sapiens	NMNAT1	NMNAT2	NMNAT3
M. musculus	Nmnat1	Nmnat2	Nmnat3
C. porcellus	Nmnat1	Nmnat2	Nmnat3
C. lupus	NMNAT1	NMNAT2	NMNAT3
G. gallus	NMNAT1	NMNAT2	NMNAT3
B. taurus	NMNAT1	NMNAT2	DAA33127
D. rerio	nmnat1	nmnat2	nmnat3
X. tropicalis	nmnat1	nmnat2	
C. elegans	F26H9.4	W06B3.1	
D. melanogaster	Nmnat		
S. cerevisiae	NMA1/NMA2		

[a] Data curated from the Ensembl, EggNOG, NCBI Orthologs, and Homologene databases (accessed January 2020).