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遗传编码荧光探针监测NADPH代谢

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Genetically encoded fluorescent sensor for monitoring NADPH metabolism

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还原型烟酰胺腺嘌呤二核苷酸磷酸(nicotinamide adenine dinucleotide phosphate, NADPH)常被视为“抗氧化的辅因子”, 通过谷胱甘肽还原酶系统和硫氧还蛋白还原酶系统清除活性氧; 同时, 它还是脂质与核苷酸等生物合成反应的重要还原力来源, 并在营养供给、氧化还原稳态与信号转导之间发挥枢纽作用, 参与肿瘤、代谢性疾病及免疫相关过程等多种生理病理事件[1,2]. 在细胞代谢网络中, 转氢反应、磷酸戊糖途径、一碳代谢及氧化脱羧等为NADPH提供还原当量, 而谷胱甘肽还原酶系统和硫氧还蛋白还原酶系统以及脂肪酸、胆固醇等合成代谢则是其主要去路[3](图1). 然而, 长期以来缺乏能够在活细胞、亚细胞水平实时监测NADPH的工具, 限制了NADPH代谢调控和急性髓系白血病机制的相关研究.

早期研究细胞内还原力时, 常借助NAD(P)H的自发荧光成像, 可检测细胞内NAD(P)H的总体水平. 随后, 荧光寿命成像可在一定程度上区分NADH和NADPH, 但其定量精度仍受限制[4]. NADPH遗传编码荧光蛋白探针iNap(indicator for NADPH)的开发, 真正推动了细胞内NADPH代谢的“可视化”, 并首次实现NADPH在活细胞、亚细胞水平的动态追踪与机制解析. 我们团队开发了对NADPH具有不同亲和力的高性能系列探针iNap1~iNap4, 展现出对NADPH高特异性、

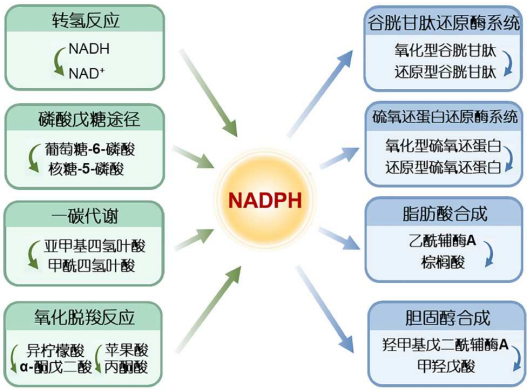


图 1 (网络版彩色)NADPH在细胞代谢网络中的作用. NADPH主要由转氢反应、磷酸戊糖途径、一碳代谢及氧化脱羧生成, 并在谷胱甘肽还原酶系统和硫氧还蛋白还原酶系统以及脂肪酸、胆固醇等合成代谢过程中提供还原力

Figure 1 (Color online) NADPH plays a central role in cellular metabolic networks. NADPH is primarily generated through the transhydrogenation, the pentose phosphate pathway, one-carbon metabolism, and the oxidative decarboxylation, and NADPH serves as a key reducing equivalent in the glutathione reductase system, the thioredoxin reductase system, as well as in anabolic processes such as fatty acid and cholesterol synthesis

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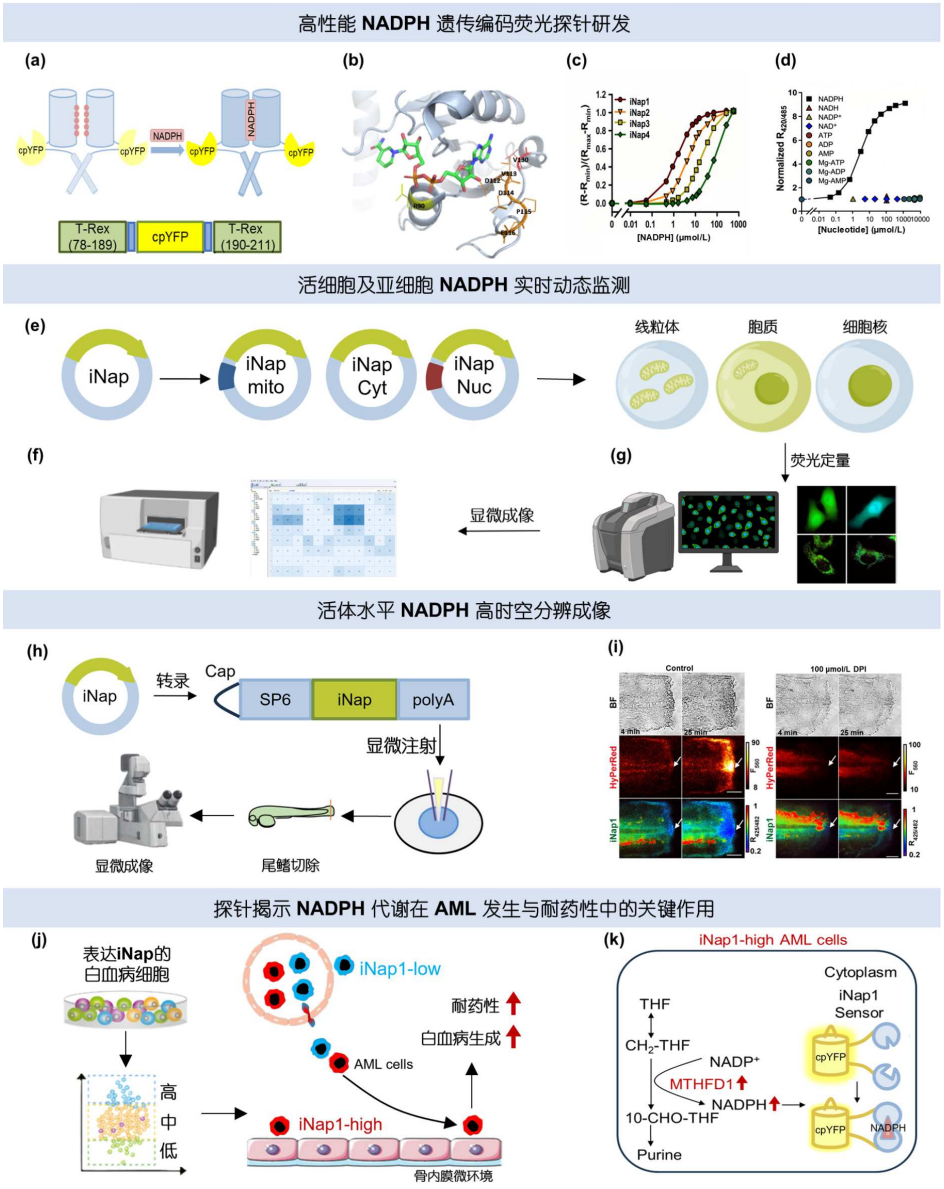


图 2 (网络版彩色)NADPH遗传编码荧光探针的开发与应用. (a) iNap探针的原理示意图; (b) T-Rex配体结合口袋及关键残基示意图; (c) iNap1~iNap4对NADPH的滴定曲线; (d) iNap探针对NADPH及其类似物的滴定曲线; (e) 将iNap探针表达在线粒体、胞质或细胞核中, 可用(f)酶标仪定量检测和(g)荧光显微成像活细胞及亚细胞内NADPH的动态变化; (h) 将iNap克隆至含SP6启动子的体外转录载体中, 将转录获得iNap探针mRNA显微注射至斑马鱼胚胎, 在幼鱼中表达并进行活体荧光成像; (i) 在斑马鱼幼鱼中共同表达iNap1和HyPerRed, 观察伤口处NADPH和H_2O_2的动态变化; (j) 在AML细胞中表达iNap1探针并根据探针荧光比值区分不同NADPH水平的细胞亚群, iNap1-high亚群更倾向定位于骨内膜微环境, 且具有更强的白血病生成能力和耐药性; (k) 在iNap1-high的AML细胞中, MTHFD1介导的一碳代谢通过促进NADPH生成, 维持较高的胞质NADPH水平(使用BioRender.com制图)

Figure 2 (Color online) Development and applications of genetically encoded NADPH fluorescent sensor. (a) Schematic of the iNap sensor design; (b) schematic illustration of the T-Rex ligand-binding pocket and key residues; (c) NADPH titration curves for iNap1–iNap4; (d) titration curves of the iNap sensor with NADPH and its analogs; (e–g) expression of iNap sensors in the mitochondria, cytosol, or nucleus enables quantitative detection of NADPH changes in living cells and subcellular compartments by microplate reader assays (f) and fluorescence imaging (g); (h) iNap was cloned into an *in vitro* transcription vector containing an SP6 promoter, and the transcribed iNap mRNA was microinjected into zebrafish embryos for expression in larvae and *in vivo* fluorescence imaging; (i) co-expression of iNap1 and HyPerRed in zebrafish larvae reveals dynamic changes in NADPH and H_2O_2 at the wound site; (j) expression of iNap1 in AML cells enables discrimination of cell subpopulations with different NADPH levels based on the sensor's fluorescence ratio; the iNap1-high subpopulation preferentially localizes to the endosteal niche and exhibits enhanced leukemogenic potential and drug resistance; (k) in iNap1-high AML cells, MTHFD1-mediated one-carbon metabolism maintains high cytosolic NADPH levels by promoting NADPH production (Created with BioRender.com)

反应迅速且可逆等优势；在不同生理病理条件下，可适配不同细胞类型与代谢状态，支持对细胞质、线粒体、细胞核等区室乃至活体水平的NADPH进行原位实时监测，从而解析NADPH在不同区室与不同刺激条件下的变化规律[5~8].

这项研究以成功开发的NAD(H)荧光探针SoNar(sensor of NAD(H) redox)为基础[9]. 基于氧化还原感应型转录抑制蛋白(redox-sensing transcriptional repressor, Rex)的三维结构信息，我们首先对NAD(H)结合口袋进行理性设计，其次对磷酸基团识别相关的位点进行突变筛选，获得对NADPH具有高选择性的原型探针(图2(a, b)). 随后系统调节连接肽的长度与氨基酸组成，并对结合口袋残基进行组合筛选，在大量突变体中兼顾响应幅度、特异性、亲和力与荧光亮度等因素，最终得到覆盖不同浓度区间的系列探针iNap1~iNap4. 蛋白滴定显示，探针对NADPH的亲和力呈现梯度分布，iNap1~iNap4对NADPH的解离常数分别约为2、6、25和120 μmol/L(图2(c))，能够覆盖不同细胞与组织中NADPH生理病理浓度变化范围. iNap探针对NADPH表现出高度选择性，并具有出色的抗干扰能力和反应迅速可逆等优势(图2(d)). 考虑到荧光蛋白对pH的固有敏感性，同步构建了不结合NADPH的iNapc作为pH对照探针，用于校正复杂生理病理条件下pH波动导致的信号扰动，从而更可靠地实现活细胞与亚细胞水平的NADPH动态成像[8].

通过将荧光探针iNap与线粒体、细胞核等定位信号肽相连接，可获得能精准定位于线粒体或细胞核的探针版本，从而实现对相应细胞区室内NADPH的高分辨成像(图2(e, f)). 我们利用分别靶向胞质与线粒体基质的iNap1和iNap3探针，检测了细胞内游离NADPH的水平(图2(g)). 结果显示，HeLa细胞线粒体基质内的NADPH水平显著高于细胞质，二者相差约10倍. 进一步研究发现，胞质中的烟酰胺腺嘌呤二核苷酸激酶(NAD kinase, NADK)可通过调控$NADP^+$的水平来调节胞质中NADPH的含量；调控胞质中的NADK活性，还能进一步引起线粒体基质中NADPH含量的同步变化[8].

本研究利用胚胎显微注射系统，将NADPH探针iNap1与过氧化氢探针HyPerRed的mRNA共同注入斑马鱼受精卵，使探针在发育的幼鱼体内表达，从而在组织水平上实现了NADPH与氧化应激信号的同步成像(图2(h)). 将幼鱼尾鳍切除后，伤口处的HyPerRed信号迅速增强，同时iNap1信号快速降低. 加入NADPH氧化酶抑制剂二苯碘鎓(diphenyleneiodonium, DPI)后，HyPerRed信号的增强与iNap1信号的降低均被显著抑制，表明局部NADPH的快速消耗与活性氧生成密切相关(图2(i))[8]. 这种多探针联用策略可在活体中同时检测还原力的消耗和氧化产物的累积，为解析复杂生理病理过程中的氧化还原动态变化提供了更为直接的技术支持.

利用高性能的NADPH荧光蛋白探针iNap，我们发现急性髓系白血病(acute myeloid leukemia, AML)细胞表现出明显的NADPH代谢异质性，可以分成不同亚群. 其中由iNap1指示的NADPH水平高的细胞亚群富集了更多功能性的白血病起始细胞，它们不但细胞增殖能力更强，而且倾向于驻留在骨内膜中，并且对AML治疗用药甲氨蝶呤具有更强的耐药性(图2(j)). 进一步深入地探究了NADPH代谢在AML细胞命运调控中的重要作用，发现亚甲基四氢叶酸脱氢酶催化的一碳代谢是AML细胞NADPH的重要来源，阻断一碳代谢可以抑制肿瘤生成能力，并降低了对甲氨蝶呤的耐药性(图2(k)). 利用自主研发的NADPH探针iNap和过氧化氢探针HyPerion，我们证明了高的还原力和低的活性氧在急性髓系白血病中的重要作用和致病机理，也为开发新的干预治疗靶点奠定了理论基础[6,10]. 近年来，iNap1和iNap3也被用于监测血管内皮细胞衰老过程中胞质与线粒体NADPH的动态变化[7]，进一步体现了iNap系列遗传编码荧光探针在活细胞NADPH动态监测中的重要价值.

NADPH作为细胞内的还原力，在生理或病理条件下发挥重要作用. 遗传编码荧光探针iNap，不仅可用于活细胞、亚细胞及在体NADPH的监测成像，也可用于急性髓系白血病的机制研究. 此外，单一指标难以刻画复杂代谢网络，未来可推动多探针正交联用，在同一细胞内同步监测多种代谢物与氧化还原水平，构建多参数表型体系，从而更系统地解析疾病发生发展与耐药复发机制，并为代谢靶向干预与联合治疗提供依据[11~13]. 与此同时，本课题组已围绕细胞内关键代谢物烟酰胺腺嘌呤二核苷酸(NAD(H))[9]、过氧化氢[5,10]、乳酸[14~16]、精氨酸[17,18]等，研发了一系列高性能遗传编码荧光探针，在细胞代谢荧光探针检测领域形成了国际领先的技术优势与全球竞争力. 这些研究成果实现了对多个代谢过程的“可视化、可量化、可动态追踪”，并为持续推动疾病机制研究的深入与药物筛选体系的优化提供了重要的技术支撑.

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