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1 Quinolimide-based peptide biosensor for probing p25 in vitro and in living

- 2 cells
- 3 Francisco Fueyo-González^{a,e}, Rosario Herranz^a, Simona Plesselova^b, Maria D. Giron^b, Rafael
- 4 Salto^b, Jose Manuel Paredes^c, Angel Orte^c, May C. Morris^{d,*}, and Juan A. González-Vera^{a,c,d,*}
- 5 ^aInstituto de Química Médica (CSIC). Juan de la Cierva 3, 28006 Madrid, Spain.
- ^bDepartamento de Bioquímica y Biología Molecular II, Facultad de Farmacia, Universidad de Granada.
 Campus Cartuja, 18071, Granada, Spain
- 8 ^cDepartamento de Fisicoquímica, Unidad de Excelencia de Química Aplicada a Biomedicina y Medioambiente,
- 9 Facultad de Farmacia, Universidad de Granada, Campus Cartuja, 18071, Granada, Spain.
- ^dInstitut des Biomolécules Max Mousseron, IBMM-UMR 5247, Université de Montpellier, Faculté de
 Pharmacie, 15 Av. Charles Flahault, 34093 Montpellier, France.
- 12 *Corresponding authors E-mail addresses: gonzalezvera@ugr.es (J. A. González-Vera);
 13 may.morris@umontpellier.fr_(M. Morris)

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15 ABSTRACT

16 CDK5 kinase is activated through interactions with different partners including the p35/p25 regulators. 17 Active CDK5 plays a key role in several neuronal functions and its hyperactivity contributes to a 18 variety of neurodegenerative processes and several human cancers, in particular glioblastomas and 19 neuroblastomas. In order to probe partners that interact with CDK5, we designed and synthesized 20 fluorescent quinolimide-labelled peptides derived from the C-helix of CDK5, which were 21 implemented to probe CDK5 partners *in vitro* and by *in cellulo* imaging in U87 glioblastoma and N2a

^eCurrent address: Department of Medicine, Translational Transplant Research Center, Immunology Institute, Icahn School of Medicine at Mount Sinai, New York, USA.

neuroblastoma cells. Ectopic expression of a NIR fluorescent protein miRFP670 fusion with p25 (p25miRFP670) in N2a cells induced FRET between a quinolimide-labelled peptide biosensor and p25miRFP670, and fluorescence intensity was proportional to the expression level of p25, thereby
demonstrating the potential of the quinolimide-labelled peptide biosensor to report on p25 relative
abundance.

Keywords: Fluorescence probes; Quinolimide derivatives; CDK5; p25 sensors; FRET imaging;
Protein-protein interactions.

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30 1. Introduction

CDK5 is a serine/threonine kinase, member of the cyclin-dependent kinase (CDK) family, which 31 is expressed ubiquitously, but primarily in post-mitotic neurons, where it is activated by the 32 neurospecific activators p35/p25 and p39/p29 [1]. CDK5 has also been reported to interact with 33 34 several cyclin partners, including cyclins E, I and Y [2]. CDK5 is mostly present in the central nervous system, where it induces the extension of neurites, neuronal maturation, synapse formation in brain 35 36 development, synaptic plasticity, axonal guidance, neuronal development and differentiation, and is involved in autophagy regulation [3, 4]. CDK5 activation occurs after the proteolytic cleavage of p35 37 38 or p39 protein partners to p25 or p29, by calpain and stimulated by Ca²⁺, which is released into the 39 cytoplasm [5, 6]. p25 does not present sequence similarity with cyclins, but adopts a similar fold and 40 binds to CDK5 at the same interface as other CDKs bind cyclins, known as the C-helix.

41 CDK5 hyperactivity is related with the onset and development of several neurodegenerative 42 diseases, inducing neuronal death [3, 7-9]. Diverse studies have demonstrated the involvement of the 43 CDK5 hyperactivation by p25 in Alzheimer's disease, Parkinson's disease and amyotrophic lateral 44 sclerosis (ALS). In fact, CDK5 participates in phosphorylation of tau protein [9-11]. It also contributes 45 to hyperphosphorylation of α -synuclein and parkin, and to the consequent generation of Lewy bodies 46 in Parkinson's disease [12, 13], and further induces neuronal loss in ALS [14]. Recently, the 47 involvement of CDK5 in neuronal death during cerebral ischemia has also been described [15]. 48 Furthermore, numerous studies report upregulation or hyperactivation of CDK5 in several types of 49 human cancers, highlighting the importance of this kinase as a drug target for cancer therapy [3, 9, 16-50 18]. Abnormal expression or hyperactivation of CDK5 is associated with tumorigenesis and tumor progression, including cancer stem cells, by promoting proliferation, migration and angiogenesis. 51 52 CDK5 activity participates in control of the DNA damage pathway, resistance to cancer chemotherapy 53 and anti-tumor immunity [16, 17]. In particular, recent studies suggest that the expression and hyperactivity of this kinase are involved in the development and degree of aggressiveness of 54 glioblastomas and neuroblastomas [19, 20]. CDK5 is an invaluable tumorigenesis and glioma 55 56 progression biomarker [20]. Therefore, CDK5 constitutes an attractive pharmacological target in neurodegenerative disorders (Parkinson's, Alzheimer's, Huntington's and ALS) and oncology. Hence 57 58 development of a fluorescent biosensor of CDK5 partners involved in its activation would constitute an attractive means of reporting on aberrant or excessive interactions of CDK5 with its partners and its 59 60 potential dysregulation in pathological conditions.

Environment-sensitive fluorophores and in particular solvatochromic dyes have been commonly 61 62 employed in the development of biosensors for probing protein-protein interactions [21, 22]. 63 Photophysical properties (e.g. emission wavelengths, quantum yields and fluorescence lifetimes) of 64 these dyes are highly dependent on the polarity of their neighboring environment, exhibiting, in 65 general, very low fluorescence emission in water and polar solvents, in contrast to that in non-polar 66 environments. This high sensitivity to their surrounding microenvironment makes them suitable for 67 reporting on molecular interactions with high signal-to-noise ratio. Since the interfaces of protein-68 protein interactions are usually hydrophobic, solvatochromic fluorophores sense these interactions through significant increases in fluorescence, relative to their free exposure to the solvent. 69

70 In this field, we have described a new family of highly solvatochromic and fluorogenic 71 fluorophores based on a 9-methoxy-quinolimide scaffold (Figure 1, A), which demonstrated 72 sensitivity for probing partners of CDK5 in glioblastoma cells [23]. The photophysical properties of

these fluorophores have been improved by replacing the electron donor 9-methoxy group by diverse 73 74 amino groups (Figure 1, B). Among the studied amino groups, quinolimides with a methylamino or the cyclic amine azetidine showed the best photophysical properties. Specifically, 9-azetidinyl-5-75 butyl-quinolimide (Figure 1, **B**: $\mathbf{R} = n\mathbf{B}\mathbf{u}$, $\mathbf{R}^1 = azetidine$) has demonstrated high potential for *in vivo* 76 77 sensing β -amyloid aggregation in a zebrafish model, enabling to identify the appearance of different 78 types of early pre-amyloid oligomers [24]. In view of the solvatochromic and fluorogenic behavior of 79 9-methylamino and 9-azetidinyl-quinolimides, we decided to prepare bromoacetamide derivatives of 80 these fluorophores as peptide labelling reagents and to implement their use to develop a CDK5-based peptide biosensor of p25 level by FRET imaging in cultured cells. 81

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83 84 85 A: R¹ = OMe 86 **B**: $R^1 = NR^2R^3$ 87 Figure 1. General structure of quinolimide-based fluorophores. 88 89 2. Experimental Section 90 2.1. Synthetic Materials and Methods 91 Complete information on the synthesis, photophysical and NMR characterization data of quinolimides 92 **2–5** (see Scheme 1) has been included in the Supplementary Material, as well as peptide labelling and titration experiments. 93 94 2.2. Colocalization of quinolimide labelled biosensor (Pep1-K5-5) with endogenous p25 in U87 glioblastoma cells 95 A previously reported protocol was followed [23]. Pep1-CDK5 peptide labelled with 5 (Pep1-K5-96

 $97 \quad 5, 2 \ \mu M$) was added to U87 cells. Indirect immunofluorescence was carried out with rabbit polyclonal

98 anti-p25 using Alexa Fluor 647-conjugated secondary antibodies and nuclei were stained with
99 Hoechst. Cells were visualized using a Zeiss microscope with a CoolSnap camera and images were
100 obtained with MetaMorph software.

101 2.3. Generation of a eukaryotic expression vector for p25 fused to the miRFP660 fluorescent
102 protein (pmiRFP670-N-P25)

103 p25 coding sequence was amplified by PCR from plasmid GST-p25 (a gift from Harish Pant, Addgene plasmid # 24896) that encodes for a truncated version of p35 protein [25]. p25 sequence, 104 105 without the stop codon was amplified from GST-p25 by PCR using Pfu polymerase and primers 106 including a 5'BamHI and 3'EcoRI restriction sites. In addition, the forward primer included a Kozak 107 sequence plus an initiation codon (Forward primer: 5'-ggatcccaccatgGCCCAGCCCGCCG-3' and 108 Reverse primer: 5'-GaattcgCCGATCCAGGCCTAGGAG-3' where the lowercase letters correspond 109 to the restriction sites, kozak sequence and initiation codon added). The PCR fragment was subcloned 110 using BamHI and 3'EcoRI restriction sites into the BglII and EcoRI restriction sites in the pmiRFP670-N1 vector (a gift from Vladislav Verkhusha, Addgene plasmid # 79987), a eukaryotic expression 111 vector that encodes the monomeric near-infrared fluorescent protein miRFP670 [26]. The resulting 112 113 plasmid was termed pmiRFP670-N-p25 and its sequence was confirmed by automatic sequencing.

114 2.4. In cellulo probing expression levels of p25 in N2a cells using dual-color ratiometric and 115 fluorescence lifetime (FLIM) imaging

116 Murine neuroblastoma Neuro-2a cells (N2a; ATCC No. CCL-131) were cultured in Dulbecco's 117 Modified Eagle's medium (DMEM) supplemented with glutamine plus (2 mM), FBS (10 %), 118 penicillin (100 units/mL) and streptomycin (0.1 mg/mL) in a CO₂ atmosphere (5 %). Prior to 119 transfection either with plasmid pmiRFP670-N-P25 or pmiRFP670-N1, cells were seeded onto 120 coverslips in 6 well plates at a density of 2.3×10^5 cells/well for 24 h to obtain an 80–90 % cell 121 confluence. For transfection experiments, pmiRFP670-N1 and pmiRFP670-N-P25 plasmids (4.3 122 µg/well) were mixed with LP2000 (10 µL) for 30 min at room temperature (100 µL of total volume). Next, the mixture was diluted with DMEM (without FBS) to 1 mL and added to the wells. Cells were
added the polyplexes and incubated for 5 h. Afterwards medium was removed and cells were grown in
DMEM plus FBS (10 %) for 24 h. Transfected cells were used for fluorescence microscopy and image
analysis. For differentiation experiments, medium was replaced with DMEM supplemented with 1%
FBS for 24h. For the experiments using rosiglitazone, N2a cells were treated with rosiglitazone 200
µM for 24h.

129 2.5. Dual-color FLIM studies

Dual-color FLIM was performed on a MicroTime 200 system (PicoQuant GmbH) as previously described [24]. Two pulsed interleaved excitation lasers at λ_{ex} = 470 nm and λ_{ex} = 635 nm were employed and fluorescence was split into two detection channels with a 600DCXR dichroic mirror. The green channel was defined with a 550/40 bandpass filter whereas a 685/70 bandpass filter was used for red channel. FLIM imaging analyses were carried out employing SymphoTime 32 (PicoQuant GmbH) and home-coded scripts in FiJi (distribution of ImageJ [27]).

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137 **3.** Results and discussion

138 *3.1. Synthesis of peptide labelling reagents*

As shown in Scheme 1, the 9-amino quinolimides **2** and **3** were obtained by replacement of the 9methoxy group of quinolimide **1** [23] by the corresponding amine, via aromatic nucleophilic substitution [28], under microwave activated heating at 120 °C. After removal of the protecting Boc group from **2** and **3**, by treatment with TFA in CH₂Cl₂, the reaction at room temperature with bromoacetic acid bromide in the presence of DIPEA, as acceptor of the generated BrH, led to the corresponding bromoacetamides **4** and **5** with 82 and 32 % yield, respectively.



146 **Scheme 1.** Synthesis of the thiol-reactive bromoacetamides 4 and 5.

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148 3.2. Photophysical properties of bromoacetamides 4 and 5

UV absorption and fluorescence emission properties of bromoacetamide derivatives 4 and 5 were determined in dioxane, as nonpolar solvent, and water and are shown in Table 1. As expected the fluorescence emission of 4 and 5 was significantly higher in dioxane than in water, as well as their respective lifetimes. Moreover, these dyes display very large Stokes shifts (> 100 nm).

153 Table 1. Photophysical properties of the bromoacetamides 4 and 5

Compd ^a	Solvent	$\lambda_{max}{}^{abs}\left(nm\right)$	$\epsilon \left(M^{-1}cm^{-1}\right)$	$\lambda_{max}{}^{em}(nm)$	$\Phi_{ ext{F}}{}^{b}$	$\tau(ns)^c$
4	Dioxane	463	15300 ± 1000	583	0.029 ± 0.002	1.80 ± 0.01
	H ₂ O	483	11100 ± 700	617	<0.001	0.20 ± 0.02
5	Dioxane	474	14200 ± 900	583	0.120 ± 0.008	3.96 ± 0.08
	H_2O	492	11200 ± 700	623	<0.001	0.68 ± 0.06

^aMeasured in duplicate at a 12 μ M concentration. ^bQuantum yields calculated with reference to coumarin 153 (in EtOH). ^cFluorescence lifetimes obtained with $\lambda_{ex} = 485$ nm, as global fits of three fluorescence decay traces collected at λ_{max}^{em} -10 nm, λ_{max}^{em} , and λ_{max}^{em} +10 nm. The reported values are the mean of the intensity-weighted average fluorescence lifetimes at the three different wavelengths from triexponencial decay fittings. The associated error is the standard deviation of the mean.

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163 3.3. Fluorescent peptide design, labelling and in vitro study of the CDK5/p25 interaction

As shown in Figure 3, the crystal structure of the CDK5/p25 complex shows that CDK5 binds p25 164 through the C-helix (PISTVRE, in red) of CDK5 and the α helix of p25 (in yellow) [29, 30]. 165 166 Therefore, to study the interaction between the C-helix of CDK5 and its partners, we labelled a peptide 167 derived from the C-helix of CDK5 (K5 peptide, GVPSSALREICLLK [23]), which bears a single cysteine residue, with the prepared bromoacetamides 4 and 5, respectively (Figure 3c). The resulting 168 labelled peptides K5-4 and K5-5 were then titrated with a peptide derived from the interacting α helix 169 of p25 (p25-S1 peptide, KEAFWDRCLSVINLM [23]). In the absence of this peptide, the emission of 170 peptides K5-4 (Figure 4a) and K5-5 (Figure 4b) was very low; however, it increased significantly in 171 the presence of increasing concentrations of the p25-S1 peptide. Addition of p25-S1 peptide to 172 labelled peptides K5-4 and K5-5 produced hypsochromic shifts of 38 and 37 nm (from red to orange) 173 174 and 21.6- and 20.2-fold fluorescence emission intensity enhancement, respectively, indicative of an effective interaction with p25-S1. 175



Figure 3. (a and b) Cartoon representation of the CDK5/p25 complex (PDB 1UNG), in two different poses, with
the interface between CDK5 (green) and p25 (blue) mediated by contacts between the C helix of CDK5
(PSTAIRE; red), and the α helix of p25 (yellow)[30]. Color code of peptides derived from CDK5/p25 interaction
employed in this work: p25-S1 peptide in yellow, p25-S2 peptide in purple and K5 peptide in red. (c) Scheme of
peptide labelling.

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183 Binding constants, $K_{\rm d}$, were obtained through the fluorescence titration curves as described in the Supplementary Material. The K_d value for K5-4 was 420 ± 80 μ M, while K5-5 exhibited a K_d value of 184 $49 \pm 15 \mu$ M, which, importantly, represents a 8.5-fold increase in affinity compared to that of K5-4 185 and 7-fold increase respect to that of our previously reported 9-methoxy-quinolimide-based biosensor 186 187 [23]. Moreover, the titration of K5-**4** and K5-5 with unrelated an peptide (VESSDTIDNVKSKIQDKEGC [31]) (Figure S3) did not produce a fluorescence increase, 188 demonstrating that the probe is not involved in a false interaction and, therefore, highlighting that 189 190 binding of the labelled peptides is selective for the p25-S1 peptide. As a negative control, we titrated K5-5 with a peptide derived from another α helix close to the interacting α helix of p25 but not 191 directly involved in the CDK5/p25 interaction (p25-S2 peptide, CSKMLQINAD), which showed a 192 193 negligible binding (Figure 4c; $K_d > 6000 \mu$ M). Furthermore, the titration of the K5 peptide labelled with a standard fluorophore with low solvatochromic behavior, such as fluorescein (FITC), only led to 194 195 1.5-fold increase in fluorescence, with no related spectral shift (Figure S3). These results show that 196 both K5-4 and K5-5 peptides constitute useful tools for studying partners that interact with the C-helix 197 of CDK5, in particular the azetidine derivative K5-5, due to its greater brightness, longer excitation 198 and emission wavelengths, larger fluorescence lifetime and greater affinity for the peptide derived 199 from p25-S1.

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Figure 4. (a) Titration of K5-4 (5 μ M) with the p25-S1 peptide (λ_{ex} = 480 nm) and curve fit. (b) Titration of K5-5 (5 μ M) with the p25-S1 peptide (λ_{ex} = 490 nm) and curve fit. (c) Titration of K5-5 (5 μ M) with the p25-S2 peptide (λ_{ex} = 490 nm) and curve fit. (d) Titration of Pep1-K5-5 (0.2 μ M) with GST-p25 protein (λ_{ex} = 490 nm) and curve fit. Insets a-d: plots of fluorescence intensity versus concentration of the peptide. Lines represent results from non-linear curve fitting from which the binding constant, *K_d*, values included in the insets were obtained.

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3.4. Colocalization of quinolimide labelled biosensor (Pep1-K5-5) with endogenous p25 in U87

214 glioblastoma cells

Next, we investigated whether K5 labelled with the 9-azetidinyl-quinolimide **5** (K5-**5**) could be used as a probe for the *in cellulo* imaging of CDK5 partners in U87 glioblastoma cells. As expected, the K5-**5** peptide, by itself, did not penetrate into cells. Therefore, a fusion of the cell-penetrating peptide Pep1 [32, 33] with the K5 peptide was synthesized and was then labelled with bromoacetamide **5**. Prior to the *in cellulo* assay, we studied the *in vitro* interaction between labelled

peptide Pep1-K5-5 (KETWWETWWTEKKGVPSSALREIC(5)LLK) and recombinant GST-p25. The 220 221 titration of Pep1-K5-5 with GST-p25 led to a hypsochromic shift of 37 nm and 10.1-fold increase in fluorescence, with a K_d value of 30 ± 6 μ M, which indicated an effective binding to p25 protein 222 (Figure 4d). Pep1-K5-5 peptide readily penetrated into living U87 cells, and its colocalization with 223 224 endogenous p25 was investigated by fluorescence microscopy following indirect immunofluorescence 225 with a p25 antibody labelled with the Alexa Fluor 647 fluorophore in U87 cells overlaid with the 226 Pep1-K5-5 peptide. The results showed a complete overlay of the signal of Pep1-K5-5 observed 227 through the GFP filter with that of Alexa Fluor 647-labelled secondary/anti-p25 primary antibodies (Figure 5a). The calculated Pearson colocalization coefficient was 0.93 ± 0.03 (Figure 5c). In contrast, 228 229 cells which had not been treated with the Pep1-K5-5 peptide did not reveal any signal through the GFP channel (Figure 5b). Remarkably, the Pep1-K5-5 peptide led to a 6.3-fold fluorescence increase, 230 inferring it interacts with p25 in cellulo. As shown in Figure 5d, this represents a significant 231 improvement compared to our previously reported 9-methoxy-quinolimide-based CDK5 probe (Pep1-232 233 K5-A) [23].



Figure 5. Colocalization of Pep1-K5-5 with p25. (a) Indirect immunofluorescence of p25 in cells treated with Pep1-K5-5. (b) Indirect immunofluorescence of p25 in cells. Images in the green channel (GFP filter) appeared with negligible signal. (c) Graph representing the intensity in the two channels used to calculate Pearson colocalization coefficient. (d) Intensity ratio I_{GFP filter}/I_{Blue filter} of Pep1-K5-5 and the analogue 9-methoxyquinolimide labelled Pep1-K5 peptide (Pep1-K5-A) [23].

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242 3.5. Reporting different levels of p25 in cellulo via FRET between Pep1-K5-5 and ectopically
243 expressed iRFP670-p25 in N2a neuroblastoma cells

Finally, we decided to assess the potential of the 9-azetidinyl-quinolimide labelled peptide Pep1-244 245 K5-5 to report on different levels and expression patterns of the p25 protein in cultured cells. For this purpose, we engineered a FRET-based system composed by our 9-amino-quinolimide labelled peptide 246 derived from CDK5 (Pep1-K5-5), as FRET donor, and p25 protein fused to the NIR fluorescent fusion 247 protein miRFP670 (p25-miRFP670), as FRET acceptor. miRFP670 was chosen based on its spectral 248 249 properties [34], which ensure a suitable FRET pair with the 9-azetidinyl-quinolimide dye thanks to a 250 good spectral overlap. In this system, when the labelled peptide Pep1-K5-5 would bind p25miRFP670, we hypothesized the probe would become embedded in a hydrophobic environment which 251 252 would increase its fluorescence and transfer the energy to miRFP670, thereby resulting in NIR 253 emission (Figure 6), together with a decrease in the fluorescence lifetime of 5. The implementation of the proposed FRET system would formally demonstrate that the CDK5-derived peptide biosensor 254 255 indeed recognizes and responds to differences in p25 abundance in cultured cells. Moreover it would provide the opportunity to develop an *in cellulo* screen of compounds, which might interfere with the 256 257 CDK5/p25 interaction, of potential therapeutic utility.



Figure 6. Schematic representation of the FRET-based biosensor for K5/p25 interaction. Upon binding of the
 Pep1-K5-5 peptide to the p25-miRFP670 target protein, the donor dye 5 would transfer its excitation energy to
 the fluorescent fusion protein, resulting in NIR emission of the acceptor.

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With this experimental design in mind, N2a neuroblastoma cells expressing physiological levels of 263 p25 [35] were either mock-transfected or transfected with pmiRFP670-N-p25 or pmiRFP670-N1 264 265 plasmids. The N2a mock-transfected cells (cells A) as well as the pmiRFP670-N1 transfected cells (cells B) express basal, physiological levels of p25. On the contrary, the pmiRFP670-N-p25 266 267 transfected cells (cells C) overexpress p25 fused to the miRFP670 fluorescent protein. Three different 268 microscope configurations (Figure 7) were selected in order to separate the emission of the fluorescently-labelled Pep1-K5-5 peptide ($\lambda_{ex} = 470 \text{ nm}$, $\lambda_{em} = 550/40 \text{ nm}$; Green channel, Figure 7a), 269 the emission of the NIR protein miRFP670 ($\lambda_{ex} = 635$ nm, $\lambda_{em} = 685/70$ nm; Red channel, Figure 7b) 270 and the last channel corresponding to the emission due to FRET ($\lambda_{ex} = 470$ nm, $\lambda_{em} = 685/70$ nm; 271 FRET channel, Figure 7c). Importantly, we also followed the FRET process by focusing on the 272 decrease in the donor's fluorescence lifetime by using fluorescence lifetime imaging (FLIM, Figure 273 274 7d).



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277 Figure 7. Intracellular FRET-based biosensor measurements and images. Box and whisker plots representing the 278 average fluorescence intensity (a) in the green channel, (b) in the red channel, (c) in the FRET channel and (d) 279 the fluorescence lifetime of the donor dye 5 in pixels from FLIM images containing Pep1-K5-5-labelled peptide 280 in N2a cells. Boxes represent the 25 and 75%, with a horizontal bar showing the average value. Whiskers 281 correspond to the minimum and maximum values. (e) Representative dual-color FLIM images containing two 282 different types of cells, cell C (top) and cell A (below), according to p25 expression levels. The three images on 283 the left represent the fluorescence intensities from the green, red and FRET channels. The right image represents 284 the fluorescence lifetime of Pep1-K5-5.

285 As shown in Figure 7a, compared to cells expressing physiological levels of p25 (cells A and B), 286 the binding of Pep1-K5-5 to cells overexpressing p25 (cells C) resulted in a >2.5-fold increase in the 287 emission intensity of the peptide biosensor in the green channel. This increase was associated with the 288 higher concentration of p25 in cells overexpressing this protein, which upon interaction with Pep1-K5-289 **5** promoted an increase in the hydrophobicity of the local environment of the fluorescent probe and, 290 therefore, increases its fluorescence emission. Importantly, this fluorescence increase successfully 291 allowed us to distinguish between cells with different intracellular concentrations of p25. Furthermore, 292 as expected, when the red channel fluorescence was studied (Figure 7b), low fluorescence levels were 293 detected in non-transfected N2a cells (cells A), while similar levels of NIR fluorescence were detected 294 on the cells transfected either with the pmiRFP670-N1 (cells B) or pmiRFP670-N-P25 plasmids (cells 295 C). As a consequence of the energy transfer from the 9-amino-quinolimide labelled peptide Pep1-K5-5 296 to p25-miRFP670, the intensity in the FRET channel, using compound 5 excitation wavelength but selecting the far-red emission channel (Figure 7c), was higher in the population of "C" cells than in the 297 298 population of "A" cells, where FRET was not possible due to the absence of the FRET acceptor p25-299 miRFP670. Furthermore, when FRET was analyzed in the pmiRFP670-N1 transfected cells ("B" cells), the signal was absent since there was no miRFP670 protein fused to the p25 moiety available 300 301 for energy transfer from the 9-amino-quinolimide labelled peptide Pep1-K5-5 onto miRFP670.

302 The interaction of Pep1-K5-5 with the p25-miRFP670 protein not only produced an increase in 303 FRET fluorescence intensity, but more significantly produced a decrease on the fluorescence lifetime 304 of Pep1-K5-5, which is an unequivocal feature of actual FRET, and is evidenced by FLIM 305 microscopy. Figure 7d shows the fluorescence lifetime obtained from the green channel for cell 306 populations A, B and C. The fluorescence lifetime of the donor quinolimide in the Pep1-K5-5 307 biosensor approximately decreased from 4.9 or 4.5 in cells expressing physiological levels of p25, 308 cells A and B respectively, to 3.7 ns in cells C, which represents an average FRET efficiency of 0.24 309 (estimated with respect to the lifetime of Pep1-K5-5 in cells A). These results confirmed the presence 310 of FRET between Pep1-K5-5 and p25-miRFP670 in cells overexpressing p25, and, therefore, 311 demonstrated the intracellular binding between the CDK5-based peptide Pep1-K5-5 and its regulatory 312 partner p25. The significant decrease in the fluorescence lifetime of the peptide biosensor confirms the 313 utility of our FRET based system to probe the CDK5/p25 interaction and to detect ectopic overexpression of p25. As expected, in the population of "B" cells, where only miRFP670 was 314 expressed, no lifetime changes in the donor due to FRET were observed. Figure 7e shows a 315 316 representative example of a dual color FLIM experiment in which two different types of cells were 317 detected. In this example a cell is characterized by a high intensity in the red and the FRET channels 318 and a low donor lifetime (cell C), whereas the other cell shows negligible intensity in the red and the FRET channel and a high donor lifetime (cell A). This indicates different p25 levels in these two cell 319 320 populations.

321 To demonstrate the usefulness of the measurement system based on the interaction of Pep1-K5-5 with the p25-miRFP670 in physio-pathological conditions, N2a cells transfected with the pmiRFP670-322 323 N-P25 plasmid (cells C) were either differentiated to neurites or treated with rosiglitazone, and then the donor lifetime of the sensor was compared to that found in the N2a untransfected cells (cells A) 324 (Figure 8). The differentiation of N2a cells to neurites involves an increase in the endogenous levels of 325 326 p25 that translated into an enhanced CDK5 activity [36]. Therefore, an increase in the lifetime could 327 be expected due to competition between the increased levels in p25 in the differentiated neurites and 328 the p25-miRFP670 protein for binding to the Pep1-K5-5 probe. On the contrary, it has been described 329 that in neuroblastoma cells, incubation with thiazolidinediones such as rosiglitazone [37] increases the degradation rate of p25 decreasing its intracellular levels. Following the same rationale, this should 330 331 translate in a decrease in the fluorescence lifetime of C cells treated with rosiglitazone. Our results 332 confirmed that the sensor can be used to follow expression levels of p25 in physiological conditions (differentiation to neurites) or after pharmacological treatment (incubation with rosiglitazone), 333 validating our measurement system in cells cultures in response to intracellular changes of p25. 334





337 Figure 8. Intracellular FRET-based biosensor measurements in physio-pathological conditions. (a) Box and 338 whisker plots representing the fluorescence lifetime of the donor dye 5 in pixels from FLIM images containing 339 Pep1-K5-5-labelled peptide in N2a cells. Boxes represent the 25 and 75%, with a horizontal bar showing the 340 average value. Whiskers correspond to the minimum and maximum values. N2a cells were transfected with the 341 pmiRFP670-N-P25 plasmid (cells C) and were either differentiated to neurites (Cells C neurites) or treated with 342 rosiglitazone (Cells C rosiglitazone). Cells A were untransfected N2a cells. (b and c) Representative dual-color 343 FLIM images (left column: fluorescence intensity from the red channel; right column: fluorescence lifetime of 344 Pep1-K5-5) containing (b) three different types of cells, cell C differentiated to neurites (top), cell A (middle) 345 and cell C undifferentiated (below), or (c) two different types of cells, cell C treated with rosiglitazone (top) and 346 cell A (below), according to p25 expression levels.

348 4. Conclusions

The results herein described illustrate the remarkable potential of highly solvatochromic and 349 350 fluorogenic 9-(substituted amino)-quinolimide-based fluorophores, and particularly 9-azetidinylquinolimides, as tools for fluorescence peptide labelling, and to obtain biosensors of cell cycle 351 regulators. Specifically we describe the design and synthesis of CDK5-based peptide biosensors 352 353 labelled with 9-methylamino- or 9-azetidinyl-quinolimides, which showed in vitro µM affinity for 354 p25, the regulatory partner of CDK5. We have further shown that the 9-azetidinyl-quinolimide labelled biosensor (Pep1-K5-5) colocalized with p25 in U87 glioblastoma cells. Finally we have 355 developed a FRET strategy combining our peptide biosensor and ectopic expression of the NIR 356 357 fluorescent miRFP670-p25 fusion protein, which has enabled to formally demonstrate the interaction 358 between the Pep1-K5-5 biosensor and ectopically expressed miRFP670-p25 in neuroblastoma N2a

359	cells. Our studies have shown that the FRET response is correlated with p25 levels. Moreover, the
360	implementation of this system opens the door to its use for the in cellulo screening of libraries of
361	compounds for identification of CDK5/p25 modulators of therapeutic interest.
362	
363	Conflict of interest
364	The authors have no conflicts of interest to declare.
365	
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374	Supplementary Material
375	Supplementary material to this article can be found online at
376	
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