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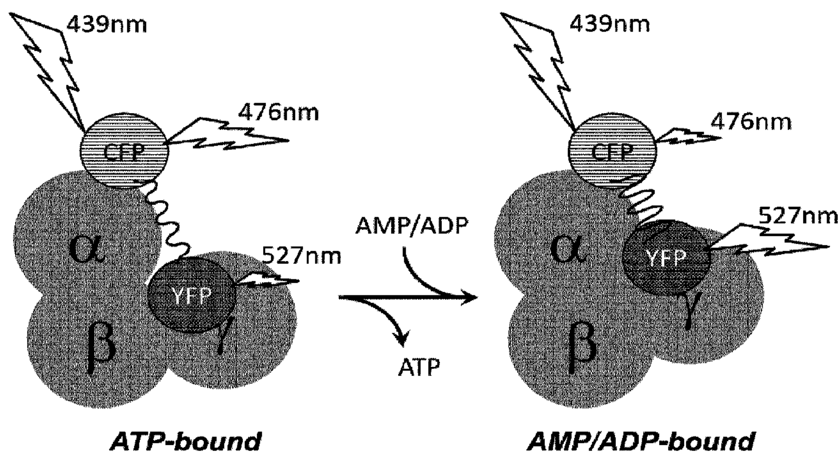

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The present invention deals with heterotrimeric AMP-activated protein kinase (AMPK) comprising a fluorophore pair wherein the conformational change can be measured by FRET. It represents an advanced tool to screen and identify AMPK interactors in vitro and in cells in vivo. Such invention can also be considered as a reporter of the cellular energy status as it allows the spatiotemporal monitoring, in situ, of fluctuations in the ratio of AMP and ADP versus ATP.

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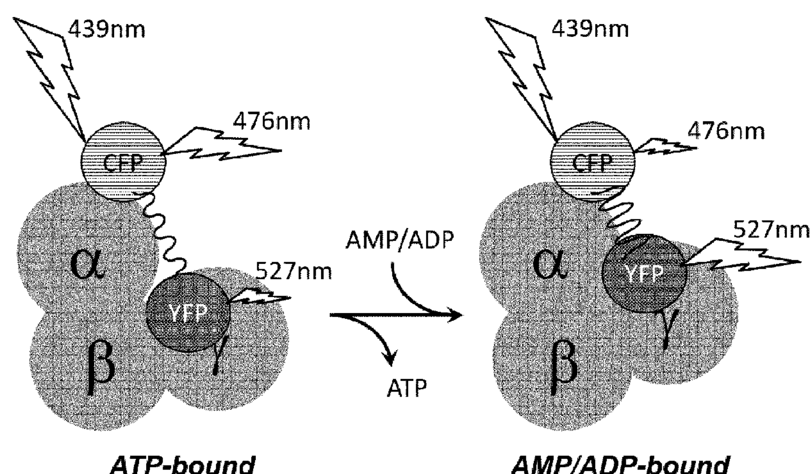
FIG. 1



(57) Abstract: The present invention deals with heterotrimeric AMP-activated protein kinase (AMPK) comprising a fluorophore pair wherein the conformational change can be measured by FRET. It represents an advanced tool to screen and identify AMPK interactors *in vitro* and in cells *in vivo*. Such invention can also be considered as a reporter of the cellular energy status as it allows the spatiotemporal monitoring, *in situ*, of fluctuations in the ratio of AMP and ADP versus ATP.

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FLUORESCENT AMP-KINASE BIOSENSORS

Genetically encoded optical biosensors allow a real time readout of molecular or cellular events with potentially high spatial and temporal resolution. They are the tool of choice for high throughput screening and quantitative studies on distribution and concentration changes of ions and metabolites in living cells. Such sensors allow multi-scale analysis in space and time that is essential for modern systems biology approaches and advanced understanding of both healthy and diseased physiological states. Optical biosensors are also adequate tools for high throughput screening of compounds and treatments *in vitro* or in cells *in vivo*.

AMP-activated protein kinase (AMPK) is a central energy sensor and regulator that monitors and responds to variations in the cellular AMP:ATP and ADP:ATP ratio (Hardie et al., Annu Rev Biochem 67:821, 1998). Upon activation of AMPK, the kinase phosphorylates an ever increasing panel of substrates to decrease further ATP usage and to increase ATP generation by the cell. Structurally, AMPK forms a heterotrimeric complex consisting of a catalytic subunit (α) and two regulatory subunits (β and γ). This AMPK complex is evolutionarily conserved in eukaryotes from yeast to plants and mammals. Mammalian AMPK is an obligatory heterotrimer composed of different isoforms of subunits: $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, $\gamma 1$, $\gamma 2$, and $\gamma 3$ (Hardie and Hawley, BioEssays 23:1112, 2001).

AMPK is activated by phosphorylation of the α-subunit on Thr172 and small molecule activators like AMP or ADP. The latter follows three distinct, additive mechanisms; 1) direct allosteric activation (by AMP alone), 2) stimulation of Thr172 phosphorylation via upstream kinases, and 3) inhibition of Thr172 dephosphorylation via protein phosphatases (Hardie et al., Nat. Rev. Mol. Cell. Biol. 13:251, 2012).

The activation of AMPK results in many beneficial metabolic effects (Kahn et al., Cell Metab 10:15, 2005). First of all, AMPK acts as a master regulator of fat and glucose metabolism. When activated, it decreases fatty acid and cholesterol synthesis, but increases fatty acid oxidation (Carling et al., FEBS Letters 223:217, 1987). Activated AMPK also stimulates glucose transport into skeletal muscle and glycolysis, and regulates expression of key genes in fatty acid and glucose metabolism in liver (summarized in U.S. Pat. No. 7,119,205). Given these effects, pharmacological AMPK activators are intensely searched as drugs against the metabolic syndrome and type 2 diabetes (Hardie et al., Annu. Rev. Pharmacol. Toxicol. 47:185, 2007). AMPK is also involved in a number of pathways that are important for some other diseases like

neurodegenerative disorders, fibrosis, osteoporosis, or heart failure (Srivastava et al., J. Lipid Res. 53:2490, 2012; Inoki et al., Annu .Rev. Pharmacol. Toxicol. 52:381, 2012).

Further, several tumour suppressors are part of the AMPK pathway, and activated AMPK negatively regulates mammalian target of rapamycin (mTOR), a key regulator of cell growth and proliferation. AMPK activators may therefore be also useful as anti-proliferative drugs (Motoshima et al., J. Physiol. 574:63, 2013). In support, the anti-diabetic drug metformin, a weak AMPK activator, has also anti-tumor effects as revealed in meta-studies (Evans et al., BMJ 330:1304, 2005).

All current anti-diabetic drugs (e.g. metformin, glitazones) are known as moderate AMPK activators, activating the kinase indirectly by inhibition of mitochondrial activity and a subsequent drop in ATP/AMP ratios, and having a number of other pleiotropic effects. Compounds that activate AMPK directly may have benefits for treating a variety of diseases mentioned above.

The activation mechanisms of AMPK are complex and not yet entirely understood. However, it is accepted now that during allosteric activation, binding of AMP or ADP to the γ subunit involves a conformational change within the AMPK heterotrimer that activates the catalytic α subunit (Riek et al., J. Biol. Chem. 283:283, 2008; Zhu et al., Structure 19:215, 2011; Chen et al., Nature Struct. Mol. Biol. 19: 716, 2012).

The present invention deals with a heterotrimeric AMPK construct comprising a fluorophore pair, allowing detection and/or measurement of conformational changes of the kinase complex. In a more specific aspect, the heterotrimeric AMPK construct of the invention allows the detection and/or the measurement of the allosteric activation of the kinase.

The present invention describes the engineering and the use of heterotrimeric AMPK constructs. Such fluorescent biosensors are able to monitor the direct effect of adenine nucleotides (AMP, ADP, ATP) and also other allosteric activators on AMPK conformation and activation.

These biosensors consist in AMPK subunits tagged directly or via intervening spacer sequences to a fluorophore pair (Figure 1). A particular aspect of the invention is the way of placing the fluorescent tags on the AMPK heterotrimer to translate conformational changes induced by allosteric AMPK activators into an exploitable change in fluorescence resonance energy transfer (or Foerster resonance energy transfer, FRET) or potentially in fluorescence quenching. As such, the present invention deals with the use a FRET signal to detect conformational changes of the AMPK complex.

The present invention deals in particular with a heterotrimeric AMP-activated protein kinase (AMPK) construct comprising or consisting in an α-subunit, a β-subunit, a γ-subunit, mutants, or fragments thereof and a fluorescent dye pair tagging at least one of said α-subunit, β-subunit or γ-subunit, said fluorescent dye pair being placed to allow detection of conformational changes within the AMPK construct.

By "AMP-activated", it is meant in the sense of the present invention that the AMPK responds to variations in the intracellular levels of adenosine monophosphate.

AMP activation of an AMPK can be measured for example with the AMPK constructs according to the present invention. In a cell in which the energy reserves are depleted, *i.e.* in which the concentration in AMP is high, the activation of AMPK by AMP or ADP will result in an increase in fluorescence resonance energy transfer. When the energy reserves of the cell increases, AMPK activation is decreased and the fluorescence resonance energy transfer signal declines proportionally to AMPK inactivation.

It is now well-established that yeast SNF-1 is not activated by AMP, but responds to variations in the glucose levels in the microorganism. The AMP-activated AMPK constructs according to the present invention are therefore not yeast SNF-1.

By "mutant", it is meant in the sense of the present invention that the wild-type AMPK is engineered by deletion, addition of amino-acids and/or mutation in the amino-acid sequence. Said α, β and/or γ subunits mutants are chosen in such a way that the AMPK retains its kinase activity and the regulatory domains, such as CBS1, CBS3 or the alpha/beta activation site, still allow allosteric activation of AMPK.

By "fragments", it is meant in the sense of the present invention that the α, β and/or γ subunits do not contain the entire amino-acid sequence of the wild type subunit. Some parts of a protein can be removed as they are not involved in regulation and/or activity. Said α, β and/or γ subunits fragments are chosen in such a way that the AMPK retains its kinase activity and the regulatory domains, such as CBS1 or CBS3, still allow allosteric activation of AMPK.

By “detection of conformational changes within the AMPK construct”, it is meant in the sense of the present invention that the FRET signal resulting from the conformational changes induced by activation of the AMPK is measured using appropriate devices, well known to those skilled in the art.

5

By “said fluorescent dye pair being placed to allow detection of conformational changes within the AMPK construct”, it is meant in the sense of the present invention that the fluorophores of the fluorescent dye pair are localized on one or more subunits in such a way that upon binding of AMP, ADP or any allosteric activator to
10 the AMPK construct, a FRET signal is observed. Without wishing to being bound by this theory, it appears that the α-subunit C-terminus approaches the C-termini of the β- and γ-subunits upon allosteric activation. The fluorophores of the fluorescent dye pair may therefore be inserted in a position close to said C-termini or at said C-termini, either directly, or after engineering of said C-termini.

15

The AMP-activated AMPK according to the present invention is a metazoan AMPK, in particular a mammalian AMPK. In particular, said mammalian AMPK is selected from the group of murine, simian, equine, human, bovine and ovine AMPK.

20 The AMP-activated AMPK according to the present invention may also be a chimeric AMPK in which the α, β and γ subunits come from two or more different metazoans, advantageously selected from mammals, in particular from mice, rat, human, bovine and ovine.

25 In contrast to yeast SNF-1, AMP-activated AMPKs exist in the form of a stable trimer, whether in activated or inactivated form. The AMPK constructs according to the present invention are constitutively stable heterotrimers.

By “constitutively stable heterotrimer”, it is meant in the sense of the present
30 invention that the AMPK construct is constitutively composed of three subunits, under any physiological condition, and irrespective of analyzed in native tissue or as recombinantly expressed in different hosts. The metazoan, in particular mammalian AMPK protein is such a stable (or constitutive) heterotrimer, since it is purified from tissue or recombinantly expressing bacteria (or other hosts) always in form of
35 heterotrimers which are stable during different purification steps and extended storage times. Metazoan AMPK differs from yeast SNF1 in that subunits of the SNF1

complex can exist as monomers, can be expressed individually and assembled into binary complexes in vitro. (e.g., Elbling et al., 2006 Subunits of the Snf1 Kinase Heterotrimer Show Interdependence for Association and Activity Journal of Biological Chemistry 281(36):26170-26180).

5

The heterotrimeric AMPK of the invention comprises one AMPK α subunit that is either α1 or α2 or a fragment thereof, one β subunit that is either β1 or β2 or a fragment thereof, and one γ subunit that is either γ1 or γ2, γ3, or a fragment thereof.

10 The α, β and γ subunits of the invention may originate from any organism such as mice, rat, human, bovine or ovine. Any combination of the subunits may be done as long as the subunits form a heterotrimeric functional AMPK.

An advantageous combination of subunits may be selected from the group 15 consisting of rat α1, human β1 (SEQ ID NO: 99) and rat γ1 (SEQ ID NO: 15) ; rat α1, human β1 and rat γ2 ; rat α1, human β1 and rat γ3 ; rat α1, human β2 and rat γ1; rat α1, human β2 and rat γ 2; rat α1, human β2 and rat γ 3; rat α2 , human β1 and rat γ 1; rat α2, human β1 and rat γ 2; rat α2, human β1 and rat γ 3; rat α2, human β2 and rat γ 1; rat α2, human β2 and rat γ 2; and rat α2, human β2 and rat γ 3, mutants or fragments of any of the 20 foregoing subunits.

In one particular embodiment, the AMP-activated AMPK is a chimeric AMPK comprising or consisting in rodent, in particular rat, α and γ subunits and human β subunit. Preferably, said chimeric AMPK consists of rat α2 and γ1 subunits and human β2 subunit.

25

In another particular embodiment, the AMP-activated AMPK is a chimeric AMPK comprising or consisting in rodent, in particular rat, α and γ subunits and human β subunit. Preferably, said chimeric AMPK consists of rat α2 and γ1 subunits and human β1 subunit.

30 The fluorescent dye pair may be any fluorescent dye pair known to those skilled in the art, advantageously chosen among Förster (or fluorescence) resonance energy transfer (FRET) pairs.

Within the meaning of the invention, the fluorophore pair (or donor and acceptor 35 pair) may be a small molecule dye or chosen among genetically encoded fluorescent proteins, such as those derived from green fluorescent protein (GFP), like

CFP (SEQ ID NO: 53) / YFP (SEQ ID NO: 51), mseCFPΔ11 (SEQ ID NO: 67) /cpVenus (SEQ ID NO: 55) or derivatives thereof.

In a particular embodiment, the fluorescent dye pair consists in genetically encoded fluorescent proteins.

The genetically encoded fluorescent proteins may in particular be chosen from the group consisting in blue Fluorescent Proteins such as T-Sapphire, cyan Fluorescent Proteins such as eCFP, mCFP, CyPet, Cerulean, mTFP1 (Teal), green Fluorescent Proteins such as EGFP, AcGFP, TurboGFP, Emerald, Azami Green, yellow Fluorescent Proteins such as EYFP, Topaz, Venus, mCitrine, YPet, PhiYFP and Orange and Red Fluorescent Proteins, such as Kusabira Orange, mOrange, tdTomato, DsRed-Monomer, mTangerine, mStrawberry, mRFP1, mCherry, mRaspberry, mPlum, provided that the actual emission and absorption spectra of the fluorophores overlap.

Advantageously, said genetically encoded fluorescent proteins consist in GFP or a GFP derived protein, or constructs thereof, preferably chosen from among CFP / YFP and $mseCFP_{\Delta 11}$ / cpVenus.

The fluorophore pair can be inserted in principle anywhere within the heterotrimeric AMPK. This includes fusion of fluorescent protein derivatives to the C- or N-termini of the AMPK subunit coding sequence (genetically encoded sensor), or chemical addition of a fluorescent dye pair to reactive residues within the subunits (e.g. cysteines).

In an advantageous embodiment, at least two of the α-subunit, β-subunit and γ-subunit of the AMPK construct are tagged with the fluorescent dye pair. More advantageously, two of the α-subunit, β-subunit and γ-subunit are tagged with the fluorescent dye pair.

By "two of the α-subunit, β-subunit and γ-subunit of the AMPK construct are tagged with the fluorescent dye pair", it is meant in the sense of the present invention that one fluorophore of the fluorescent dye pair is attached to one subunit and the other fluorophore of the fluorescent dye pair is attached to one of the remaining subunits.

In a particular embodiment, the α-subunit and either the β or the γ subunit are tagged with the fluorescent dye pair.

It is advantageous that the α-subunit is tagged with one fluorophore of the
5 fluorescent dye pair at the C-terminus. It is also advantageous that the C-terminus of the β or the γ subunit is tagged by the fluorophore.

Preferably, the α subunit is tagged at the C-terminus and either the β or the γ subunit is tagged at the C-terminus.

10

More preferably, the α subunit is tagged at the C-terminus and either the β or the γ subunit is tagged at the C-terminus with genetically encoded fluorescent proteins, such as those cited above, in particular derived from green fluorescent protein (GFP), like CFP /YFP, mseCFP $_{\Delta 11}$ / cpVenus or derivatives thereof. Even more
15 preferably, said genetically encoded fluorescent protein consists in GFP or a GFP derived protein, or constructs thereof, preferably chosen from among CFP / YFP and mseCFP$_{\Delta 11}$ / cpVenus. According to a specific aspect of the invention, the heterotrimeric AMPK comprises α, β and γ subunits, wherein the α subunit is tagged with CFP or mseGFPΔ11 at the C-terminus and the β or the γ subunit is tagged with
20 YFP or cpVenus at the C-terminus.

The AMPK construct, *i.e.* one or more among the α, β and γ subunits, can be further engineered to improve the FRET response, for example by deletion, addition or substitution of sequences.

25

Further constructs wherein the linker sequence between AMPK subunit and dye is altered in length or its flexibility is reduced are **therefore** also parts of the invention.

Deletion includes the removal of amino-acids, in particular at the termini of one
30 or more subunits.

In embodiments in which one fluorophore of the fluorescent dye pair is located on a terminus of a subunit, *i.e.* on the C-terminus or the N-terminus, the amino-acids are preferably deleted between said terminus and the fluorophore. Said deletion may
35 be done on one or two of the tagged subunits.

Addition includes grafting additional amino-acids within one or more of the subunits. Advantageously, amino-acids are added at the terminus of one or more subunits, *i.e.* on the C-terminus or the N-terminus.

5 Amino-acids may be added for rigidifying the structure, in particular for amplifying the FRET signal observed.

In embodiments where a fluorophore of the fluorescent dye pair is located on a terminus of a subunit, *i.e.* on the C-terminus or the N-terminus, the amino-acids are 10 preferably added between said terminus and the fluorophore.

In one particular embodiment, the amino-acids added between the terminus and the fluorophore fold into a rigid α-helix. The amino-acid sequence is in particular SEQ-ID NO: 1.

15

Examples of an α subunit in which a rigid α-helix added between said α subunit and the fluorophore are SEQ ID NO: 84 and SEQ ID NO: 85.

In a particular embodiment, the α subunit is engineered by deletion of the C-20 terminal amino-acids and addition of a rigid α-helix. Preferably, the β2 or γ1 tagged with the second fluorophore of the fluorescent dye pair is engineered by deletion of amino-acids at the terminus and said second fluorophore is attached to said β2 or γ1 subunit at the new terminus.

25 Substitution in the amino-acid sequence includes replacement of an amino-acid by another. Said modification may be introduced anywhere within one or more subunits, provided that the AMPK retains its activity and that the allosteric regulatory domains remain functional.

30 By "the allosteric regulatory domains remain functional", it is meant in the sense of the present invention that the domain(s) of the subunits to which the allosteric interactor, such as e.g. AMP or ADP, bind enables an activation of AMPK.

The inventors have for example discovered that the CBS3 domain of the γ1 35 subunit is mandatory for allosteric activation of AMPK. Indeed, replacing valine 275 and leucine 276 by glycine residues (SEQ ID NO: 33 and SEQ ID NO: 37), thereby

altering AMP binding to the CBS3 regulatory domain, results in a lack of activation by AMP, as evidenced by the absence of a FRET signal.

Such an AMPK in which the CBS3 regulatory domain has been inactivated can however be used as a negative control, either *in vitro* or *ex vivo*, to validate the allosteric activation of AMPK *via* CBS3 binding.

Examples of such mutations are for example:

- the replacement of Theonine 172 of the α2 subunit, in particular with alanine (such as SEQ ID NO: 25) or aspartic acid (such as SEQ ID NO: 27),
- the replacement of amino-acids in the CBS domains, such as, for example, serine 315 of the γ1 subunit, in particular with proline (such as SEQ ID NO: 31 and SEQ ID NO 39), or
- replacement of leucine 128 with aspartic acid and valine 129 with aspartic acid in the γ1 subunit (such as SEQ ID NO: 29 and SEQ ID NO 35).

In a particular embodiment, the AMPK construct consists in α2, β2 and γ1 subunits.

In the AMPK in which the subunits are α2, β2 and γ1:

- the α2 subunit may be tagged at its N-terminus, for example with eCFP (such as SEQ ID NO: 7) or mseCFPΔ11 (such as SEQ ID NO: 69) or at its C-terminus, for example with eCFP (such as SEQ ID NO: 5) or mseCFPΔ11 (such as SEQ ID NO: 65),
- the β2 subunit may be tagged at its N-terminus, for example with YFP (such as SEQ ID NO: 13) or cpVenus (such as SEQ ID NO: 59), or at its C-terminus, for example with cpVenus (such as SEQ ID NO: 63) or YFP (such as SEQ ID NO: 23),
- the γ1 subunit may be tagged at its N-terminus, for example with cpVenus (such as SEQ ID NO: 63), YFP (such as SEQ ID NO: 47 and SEQ ID NO: 49), eCFP (such as SEQ ID NO: 19 and SEQ ID NO: 45) or mseCFPΔ11 (such as SEQ ID NO: 73) or at its C-terminus, for example with cpVenus (such as SEQ ID NO: 61), eCFP (such as SEQ ID NO: 17), YFP (such as SEQ ID NO: 47) or mseCFPΔ11 (such as SEQ ID NO: 71),

provided that the actual emission and absorption spectra of the fluorophores overlap.

In the AMPK in which the subunits are α2, β2 and γ1, it is preferred that the α2 subunit is tagged at the C-terminus and either the β2 or the γ1 subunit is tagged at the C-terminus, in particular with genetically encoded fluorescent proteins, as defined above.

According to a specific aspect of the invention, the heterotrimeric AMPK comprises α2, β2 and γ1 subunits, wherein the α2 subunit is tagged with CFP (such as SEQ ID NO: 4) or mseGFPΔ11 (such as SEQ ID NO: 65) at the C-terminus and the β2 or the γ1 subunit is tagged with YFP (such as β2: SEQ ID NO: 11; γ1: SEQ ID NO: 21 and SEQ ID NO: 47) or cpVenus (such as β2: SEQ ID NO: 57; γ1: SEQ ID NO: 61) at the C-terminus.

In this specific aspect of the invention, the AMPK construct consists in α2, β2 and γ1 subunits, the α2 subunit is tagged with mseCFPΔ11 at the C-terminus and either the β2 or the γ1 subunit is tagged with cpVenus at the C-terminus or the α2 subunit is tagged with CFP at the C-terminus and either the β2 or the γ1 subunit is tagged with YFP at the C-terminus.

More specifically, said AMPK is selected from the following AMPK constructs:

- AMPK comprising or consisting in an α2 subunit tagged with CFP at the C-terminus, a β2 subunit tagged with YFP at the C-terminus and an untagged γ1 subunit,
- AMPK comprising or consisting in an α2 subunit tagged with CFP at the C-terminus, an untagged β2 subunit and a γ1 subunit tagged with YFP at the C-terminus,
- AMPK comprising or consisting in an α2 subunit tagged with mseCFPΔ11 at the C-terminus, an untagged β2 subunit and a γ1 subunit tagged with cpVenus at the C-terminus.

The AMPK construct may be further engineered by deletion, addition or substitution of sequences, as defined above.

In a particular embodiment, the AMPK construct consists in an α2 subunit tagged at the C-terminus, a β2 subunit tagged at the C-terminus and an untagged γ1 subunit, or a mutated γ1 subunit, as defined above wherein the C-terminus of the α2

subunit is connected to the fluorophore through a rigid α-helix, advantageously consisting of SEQ ID 1 (EEEEKKKK).

In another particular embodiment, the AMPK construct consists in an α2 subunit tagged at the C-terminus, a β2 subunit tagged at the C-terminus and an untagged γ1 subunit, or a mutated γ1 subunit, as defined above wherein the three C-terminal amino-acids of the β2 subunit are deleted and the fluorophore is connected to the engineered C-terminal amino-acid of the β2 subunit.

In a further particular embodiment, the AMPK construct consists in an α2 subunit tagged at the C-terminus, a β2 subunit tagged at the C-terminus and an untagged γ1 subunit, or a mutated γ1 subunit, as defined above wherein the C-terminus of the α2 subunit is connected to the fluorophore through a rigid α-helix, advantageously consisting of SEQ ID 1 (EEEEKKKK) and the three C-terminal amino-acids of the β2 subunit are deleted and the fluorophore is connected to the engineered C-terminal amino-acid of the β2 subunit.

In a more particular embodiment, the AMPK construct comprises at least the two following subunits:

- SEQ ID NO: 75 and SEQ ID NO: 78,
- SEQ ID NO: 82 and SEQ ID NO: 89,
- SEQ ID NO: 75 and SEQ ID NO 80
- SEQ ID NO: 82 and SEQ ID NO 93
- SEQ ID NO: 85 and SEQ ID NO: 78
- SEQ ID NO: 84 and SEQ ID NO: 89,
- SEQ ID NO: 85 and SEQ ID NO: 80
- SEQ ID NO: 84 and SEQ ID NO 93.

In specific embodiments, the AMPK construct is chosen from the group consisting of:

- SEQ ID NO: 75, SEQ ID NO: 78 and SEQ ID NO 15,
- SEQ ID NO: 82, SEQ ID NO: 89 and SEQ ID NO 15,
- SEQ ID NO: 75, SEQ ID NO: 9 and SEQ ID NO 80
- SEQ ID NO: 82, SEQ ID NO: 9 and SEQ ID NO 93
- SEQ ID NO: 85, SEQ ID NO: 78 and SEQ ID NO 15
- SEQ ID NO: 84, SEQ ID NO: 89 and SEQ ID NO 15
- SEQ ID NO: 85, SEQ ID NO: 9 and SEQ ID NO 80
- SEQ ID NO: 84, SEQ ID NO: 9 and SEQ ID NO 93

In another particular embodiment, the AMPK construct consists in α2, β1 and γ1 subunits.

In the AMPK in which the subunits are α2, β1 and γ1, it is preferred that the α2 subunit is tagged at the C-terminus and either the β1 or the γ1 subunit is tagged at the C-terminus, in particular with genetically encoded fluorescent proteins, as defined above.

According to a specific aspect of the invention, the heterotrimeric AMPK comprises α2, β1 and γ1 subunits, wherein the α2 subunit is tagged with CFP or mseGFPΔ11 at the C-terminus and the β1 or the γ1 subunit is tagged with YFP or cpVenus at the C-terminus.

In this specific aspect of the invention, the AMPK construct consists in α2, β1 and γ1 subunits, the α2 subunit is tagged with mseCFPΔ11 at the C-terminus and either the β1 or the γ1 subunit is tagged with cpVenus at the C-terminus or the α2 subunit is tagged with CFP at the C-terminus and either the β1 or the γ1 subunit is tagged with YFP at the C-terminus.

In this specific aspect of the invention, the AMPK construct consists in α2, β1 and γ1 subunits, the α2 subunit is tagged with mseCFPΔ11 at the C-terminus and either the β1 or the γ1 subunit is tagged with cpVenus at the C-terminus or the α2 subunit is tagged with CFP at the C-terminus and either the β1 or the γ1 subunit is tagged with YFP at the C-terminus.

More specifically, said AMPK is selected from the following AMPK constructs:

- AMPK comprising or consisting in an α2 subunit tagged with CFP at the C-terminus, a β1 subunit tagged with YFP at the C-terminus and an untagged γ1 subunit,
- AMPK comprising or consisting in an α2 subunit tagged with CFP at the C-terminus, an untagged β1 subunit and a γ1 subunit tagged with YFP at the C-terminus,
- AMPK comprising or consisting in an α2 subunit tagged with mseCFPΔ11 at the C-terminus, an untagged β1 subunit and a γ1 subunit tagged with cpVenus at the C-terminus.

The AMPK construct may be further engineered by deletion, addition or mutations, as defined above.

In a particular embodiment, the AMPK construct consists in an α2 subunit
5 tagged at the C-terminus, a β1 subunit tagged at the C-terminus and an untagged γ1 subunit, or a mutated γ1 subunit, as defined above wherein the C-terminus of the α2 subunit is connected to the fluorophore through a rigid α-helix, advantageously consisting of SEQ ID 1 (EEEEKKKK).

In another particular embodiment, the AMPK construct consists in an α2 subunit
10 tagged at the C-terminus, a β1 subunit tagged at the C-terminus and an untagged γ1 subunit, or a mutated γ1 subunit, as defined above wherein the five C-terminal amino-acids of the β1 subunit are deleted and the fluorophore is connected to the engineered C-terminal amino-acid of the β1 subunit.

In a further particular embodiment, the AMPK construct consists in an α2
15 subunit tagged at the C-terminus, a β1 subunit tagged at the C-terminus and an untagged γ1 subunit, or a mutated γ1 subunit, as defined above wherein the C-terminus of the α2 subunit is connected to the fluorophore through a rigid α-helix, advantageously consisting of SEQ ID 1 (EEEEKKKK) and the five C-terminal amino-acids of the β1 subunit are deleted and the fluorophore is connected to the engineered C-
20 terminal amino-acid of the β1 subunit.

In a more particular embodiment, the AMPK construct comprises at least the two following subunits:

- SEQ ID NO: 75 and SEQ ID NO: 97,
25
- SEQ ID NO: 85 and SEQ ID NO: 97.

In specific embodiments, the AMPK construct is chosen from the group consisting of:

- SEQ ID NO: 75, SEQ ID NO: 97 and SEQ ID NO 15,
- SEQ ID NO: 85, SEQ ID NO: 97 and SEQ ID NO 15.

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According to another aspect of the invention, the CFP and YFP can be replaced by any one of said molecule variants.

The present invention also deals with the nucleic acid molecule encoding the
35 trimeric AMPK and a fluorescent dye pair. The nucleic acid can be DNA.

The present invention deals particularly with the nucleic acid molecule encoding a heterotrimeric AMP-activated protein kinase (AMPK) construct comprising or consisting in an α-subunit, a β-subunit, a γ-subunit, mutants, or fragments thereof and a genetically encoded fluorescent dye pair tagging at least one of said α-subunit, β-subunit or γ-subunit, said fluorescent dye pair being placed to allow detection of conformational changes within the AMPK construct.

The nucleic acid defined above can be inserted in a vector. The vector of the invention may be an expression vector wherein the nucleic acid molecule is operatively linked to one or more control sequences allowing expression in prokaryotic and/or eukaryotic hosts. The hosts containing at least one vector or at least one nucleic acid molecule as described are further aspects of the invention.

Said host can be a bacteria, an insect, fungal, plant or animal cell and more preferably a human cell or human cell line.

In a particular embodiment, expression vectors comprising a nucleic acid sequence encoding for the α-subunit, the β-subunit or the γ-subunit is selected from the group consisting in:

- α-subunit: SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 64, SEQ ID NO: 68, SEQ ID NO: 74, SEQ ID NO: 81, SEQ ID NO: 76 and SEQ ID NO: 83,
- β-subunit: SEQ ID NO: 12, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 77 , SEQ ID NO: 86, SEQ ID NO: 88,
- γ-subunit: SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 34, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 70, SEQ ID NO: 72, SEQ ID NO: 79, SEQ ID NO: 90, SEQ ID NO: 92.

In an advantageous embodiment, the invention concerns expression vectors encoding for a pair of tagged subunits selected from the group consisting in:

- SEQ ID NO: 74 and SEQ ID NO: 77,
- SEQ ID NO: 81 and SEQ ID NO: 88,
- SEQ ID NO: 74 and SEQ ID NO 79
- SEQ ID NO: 81 and SEQ ID NO 92

- SEQ ID NO: 76 and SEQ ID NO: 77
- SEQ ID NO: 83 and SEQ ID NO: 88,
- SEQ ID NO: 76 and SEQ ID NO: 79,
- SEQ ID NO: 83 and SEQ ID NO 92,
- 5 SEQ ID NO: 74 and SEQ ID NO: 96, or
- SEQ ID NO: 76 and SEQ ID NO: 96.

In a more advantageous embodiment, the invention concerns expression vectors encoding for an AMPK construct selected from the group consisting in:

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- SEQ ID NO: 74, SEQ ID NO: 77 and SEQ ID NO 14,
- SEQ ID NO: 81, SEQ ID NO: 88 and SEQ ID NO 14,
- SEQ ID NO: 74, SEQ ID NO: 8 and SEQ ID NO 79,
- SEQ ID NO: 81, SEQ ID NO: 8 and SEQ ID NO 92,
- 15 SEQ ID NO: 76, SEQ ID NO: 77 and SEQ ID NO 14,
- SEQ ID NO: 83, SEQ ID NO: 88 and SEQ ID NO 14,
- SEQ ID NO: 76, SEQ ID NO: 8 and SEQ ID NO 79,
- SEQ ID NO: 83, SEQ ID NO: 8 and SEQ ID NO 92,
- SEQ ID NO: 74, SEQ ID NO: 96 and SEQ ID NO 14, or
- 20 SEQ ID NO: 76, SEQ ID NO: 96 and SEQ ID NO 14.

The fluorescent signal can be detected either with recombinant heterotrimeric protein *in vitro* or with transfected AMPK tagged subunits in cells *ex vivo*. The signals can be used to screen AMPK interactors (*in vitro* or in cells *ex vivo*), or for 25 quantification of cellular AMP and ADP levels in the physiologically important low micromolar range (in cells *ex vivo*).

In another aspect, the present invention deals with a method for identifying an interactor of AMPK or its concentration in a sample.

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The present invention also concerns a method for identifying an AMPK allosteric interactor and/or its concentration in a sample comprising the steps of:

a. contacting the sample with an heterotrimeric AMPK construct as defined above or a host cell as defined above,

35 b. detecting a modification of the FRET fluorescence by fluorescence techniques.

In an advantageous embodiment, the *in vitro* method for identifying an interactor of AMPK comprises the steps of:

a. contacting an AMPK with a candidate interactor,
b. detecting the fluorescence signal by fluorescence techniques,
c. comparing the fluorescence signal with the fluorescence signal under the same conditions in the absence of said candidate allosteric interactor.

Within the context of the invention, a "candidate interactor" is intended to mean any natural or synthetic small molecule.

By comparing the fluorescence signal in the presence of the test compound with the fluorescence signal in the absence of the candidate allosteric interactor, it is possible to determine if said candidate allosteric interactor interacts with the AMPK.

A FRET signal indicates that the candidate allosteric interactor activates AMPK. The absence of the FRET signal indicates that the candidate allosteric interactor does not activate AMPK.

The *in vitro* method described above may also be used for quantifying the concentration of an allosteric interactor in a sample. The intensity of the FRET signal measured by fluorescence techniques being proportional to the concentration of the allosteric interactor in the sample, comparing the intensity of the FRET signal with values of the fluorescence intensity determined under the same conditions with determined concentrations of the allosteric interactor, one can thereby determine the concentration of the allosteric interactor in the sample.

For example, the fluorescence intensity of the sample of unknown concentration in an allosteric interactor may be compared with a calibration curve established with samples of predetermined concentrations in said allosteric interactor.

A further aspect of the invention relates to an ***ex vivo*** method of screening an AMPK interactor, the method comprising:

- providing a cell culture comprising cells expressing the heterotrimeric AMPK of the invention;
- providing candidate interactor;
- contacting the cells with said candidate interactor and

- detecting a modification of the fluorescence by fluorescent techniques and more particularly detecting a FRET signal.

If an AMPK candidate interactor results in the detection of a FRET signal, either *in vitro* upon contact with the AMPK construct or *ex vivo* with a cell culture comprising cells expressing the heterotrimeric AMPK construct, then said AMPK candidate interactor can be considered an AMPK allosteric activator.Conversely, an allosteric interactor resulting in a decline of the FRET signal of the AMPK construct activated for example with AMP or ADP, can be considered an allosteric inhibitor of AMPK.

The present invention also deals with a kit for identifying the presence of an interactor of AMPK in a sample, the kit comprising a trimeric AMPK as defined above or a microarray of the invention, the reagents and instructions for use.

The present invention also deals with a kit for transfecting a cell or cell population, comprising the nucleic acid molecule encoding the trimeric AMPK and the fluorescent dye pair, the reagents and instructions for use.

Within the meaning of the invention, the allosteric interactor is a compound that interacts with the heterotrimeric AMPK leading to a conformational change that can be detected and/or measured. Said interactor can be either an inhibitor or an activator of the AMPK. Examples of already known direct activators are A-769662 or the AICAR (5'-Aminoimidazole-4-carboxamide ribonucleoside)-derivative ZMP.

In a specific embodiment of the invention, the FRET signal is used to identify an interactor of AMPK. Within a cell, the FRET signal corresponds to a physiological increase in AMP and ADP concentrations, and can thus be used as a direct readout of the cellular energy state.

The present invention further deals with the use of the AMPK construct defined above for the detection of conformational changes within AMPK.

Upon activation of the AMPK construct by an allosteric activator, such as AMP, the induced faint conformational changes are translated into a FRET signal.

The AMPK constructs according to the invention are particularly valuable, in that the FRET signal only reflects allosteric activation of the AMPK construct and not activation by other pathways, such as Thr172 phosphorylation.

5 In the sense of the present invention, allosteric activation is the regulation of enzymatic activity by binding of an effector molecule, such as adenine nucleotides or synthetic activators at a site different to the enzyme's active site. Known regulatory sites are located on the gamma subunit and at the alpha/beta interface.

10 In the sense of the present invention, the "conformational changes" are considered faint, as they lie at the detection limits of other techniques such as small angle X-ray scattering. Previous studies have shown that the difference in radius of gyration and the particle volume of the whole heterotrimer is of less than 5 % between the inactive form (no nucleotide) and the active form (AMP-bound) with no 15 change in maximal intramolecular distance, indicating minimal changes in the structure.

The liability of the AMPK construct to translate allosteric activation of AMPK into a FRET signal (or the deactivation of AMPK into fluorescence quenching) can hence be 20 used for the identification of an allosteric interactor of AMPK.

The present invention also concerns a method for detecting conformational changes within AMPK, comprising measuring a FRET signal.

25 The present invention also concerns a method for identifying an allosteric interactor of AMPK comprising detecting conformational changes within AMPK by fluorescence technique, in particular FRET.

AMPK being involved in several pathologies, the present invention also concerns 30 the use of an AMPK construct as described above for the identification of a drug useful for the treatment of metabolic syndrome, type 2 diabetes, neurodegenerative disorders, fibrosis, osteoporosis, heart failure and proliferative diseases such as cancer.

35 Most pathologies are associated with bioenergetic dysfunction, mostly concerning mitochondrial ATP production, whether this is part of the etiology of the

disease or just one of its consequences. These pathologies include, but are not limited to cardiovascular diseases, metabolic syndrome, (neuro)muscular diseases, neurodegenerative diseases, and specific forms and stages of cancer development. Bioenergetic dysfunction is buffered to a certain degree, but beyond a threshold, it leads to decreased cellular energy state, i.e. decreased ATP and increased ADP and AMP concentrations. Exactly these early changes in AMP and ADP concentration that occur in the lower micromolar concentration range, are detected by AMPK via conformational changes, leading to kinase activation that triggers compensatory adaptations of metabolism. Thus, AMPfret can detect very early a potential onset of a pathological development.

The present invention therefore also concerns the use of an AMPK construct as defined above for detecting a pathological development, including, but not limited to, cardiovascular diseases, metabolic syndrome, (neuro)muscular diseases, neurodegenerative diseases, and specific forms and stages of cancer development.

Whether the AMPK construct is used for the detection of conformational changes within AMPK construct or the identification of the interactor of AMPK, change in fluorescence resonance energy transfer or in fluorescence quenching is measured.

By transfecting a cell with the AMPK constructs according to the invention, it is possible to obtain a real-time readout of the cellular energy state. In a cell in which the energy reserves are depleted, *i.e.* in which the concentration in AMP and ADP is high, this will result in an increase in fluorescence resonance energy transfer. Conversely, when the energy reserves of the cell increases, the fluorescence resonance energy transfer signal decreases (fluorescence decline).

The AMPK constructs according to the present invention may therefore be used to study the events occurring when a cell is subjected to an external stress, for example deprivation of oxygen or oxidative stress.

In a further embodiment, said AMPK constructs according to the present invention may be transfected in a pathological cell for assessing the effect of a candidate compound on the cell.

The present invention therefore also concerns the use of a pathological cell culture comprising cells expressing the heterotrimeric AMPK of the invention for screening a drug liable of treating said pathological cell.

5 The present invention also concerns an *ex vivo* method of screening a drug candidate against a pathology associated with AMPK, the method comprising:

- providing a pathological cell culture comprising cells expressing the heterotrimeric AMPK of the invention;
- providing candidate drug;
10 - contacting the cells with said candidate drug and
- detecting a modification of the fluorescence by fluorescent techniques and more particularly detecting a FRET signal.

The FRET signal observed with a pathological cell differs from the one of a 15 healthy cell. By comparing the FRET signal of the pathological cell incubated with a candidate drug with the one of the healthy cell, one can determine if said candidate drug is liable to restore the normal functions of the cell and if said candidate drug is useful for treating and/or preventing said pathology.

20 The expression vectors, subunits and fluorophores according to the present invention are described thereafter:

Nucleic acid	**Protein**	**Description**
	SEQ ID NO: 1	EEEEKKKK, rigid α-helix
SEQ ID NO: 2	SEQ ID NO: 3	Rat α2 subunit
SEQ ID NO: 4	SEQ ID NO: 5	Rat α2 subunit tagged with eCFP at its C-terminus
SEQ ID NO: 6	SEQ ID NO: 7	Rat α2 subunit tagged with eCFP at its N-terminus
SEQ ID NO: 8	SEQ ID NO: 9	Human β2 subunit
SEQ ID NO: 10	SEQ ID NO: 11	Human β2 subunit tagged with YFP at its C-terminus
SEQ ID NO: 12	SEQ ID NO: 13	Human β2 subunit tagged with YFP at its N-terminus
SEQ ID NO: 14	SEQ ID NO: 15	Rat γ1 subunit
SEQ ID NO: 16	SEQ ID NO: 17	Rat γ1 subunit tagged with eCFP at its C-

		terminus
SEQ ID NO: 18	SEQ ID NO: 19	Rat γ1 subunit tagged with eCFP at its N-terminus
SEQ ID NO: 20	SEQ ID NO: 21	Rat γ1 subunit tagged with YFP at its C-terminus
SEQ ID NO: 22	SEQ ID NO: 23	Rat γ1 subunit tagged with YFP at its N-terminus
SEQ ID NO: 24	SEQ ID NO: 25	Rat α2 subunit tagged with eCFP at its C-terminus, threonine 172 replaced by alanine
SEQ ID NO: 26	SEQ ID NO: 27	Rat α2 subunit tagged with eCFP at its C-terminus, threonine 172 replaced by aspartic acid
SEQ ID NO: 28	SEQ ID NO: 29	Rat γ1 subunit, leucine 128 and valine 129 replaced by aspartic acid residues
SEQ ID NO: 30	SEQ ID NO: 31	Rat γ1 subunit, serine 315 replaced by proline
SEQ ID NO: 32	SEQ ID NO: 33	Rat γ1 subunit, valine 275 and leucine 276 replaced by glycine residues. Negative control
SEQ ID NO: 34	SEQ ID NO: 35	Rat γ1 subunit tagged with YFP at its C-terminus, leucine 128 and valine 129 replaced by aspartic acid residues
SEQ ID NO: 36	SEQ ID NO: 37	Rat γ1 subunit tagged with YFP at its C-terminus, valine 275 and leucine 276 replaced by glycine residues. Negative control
SEQ ID NO: 38	SEQ ID NO: 39	Rat γ1 subunit tagged with YFP at its C-terminus, serine 315 replaced by proline
SEQ ID NO: 40	SEQ ID NO: 41	Rat γ1 subunit, T7 vector
SEQ ID NO: 42	SEQ ID NO: 43	Rat γ1 subunit tagged with eCFP at its C-terminus, T7 vector
SEQ ID NO: 44	SEQ ID NO: 45	Rat γ1 subunit tagged with eCFP at its N-terminus, T7 vector
SEQ ID NO: 46	SEQ ID NO: 47	Rat γ1 subunit tagged with YFP at its C-terminus, T7 vector

SEQ ID NO: 48	SEQ ID NO: 49	Rat γ1 subunit tagged with YFP at its N-terminus, T7 vector
SEQ ID NO: 50	SEQ ID NO: 51	eYFP
SEQ ID NO: 52	SEQ ID NO: 53	eCFP
SEQ ID NO: 54	SEQ ID NO: 55	cpVenus
SEQ ID NO: 56	SEQ ID NO: 57	Human β2 subunit tagged with cpVenus at its C-terminus
SEQ ID NO: 58	SEQ ID NO: 59	Human β2 subunit tagged with cpVenus at its N-terminus
SEQ ID NO: 60	SEQ ID NO: 61	Rat γ1 subunit tagged with cpVenus at its C-terminus
SEQ ID NO: 62	SEQ ID NO: 63	Rat γ1 subunit tagged with cpVenus at its N-terminus
SEQ ID NO: 64	SEQ ID NO: 65	Rat α2 subunit tagged with mseCFPΔ11 at its C-terminus
SEQ ID NO: 66	SEQ ID NO: 67	mseCFPΔ11
SEQ ID NO: 68	SEQ ID NO: 69	Rat α2 subunit tagged with mseCFPΔ11 at its N-terminus
SEQ ID NO: 70	SEQ ID NO: 71	Rat γ1 subunit tagged with mseCFPΔ11 at its C-terminus
SEQ ID NO: 72	SEQ ID NO: 73	Rat γ1 subunit tagged with mseCFPΔ11 at its N-terminus
SEQ ID NO: 74	SEQ ID NO: 75	Rat α2 subunit tagged with eCFP at its C-terminus, C-terminal AR residues deleted
SEQ ID NO: 77	SEQ ID NO: 78	Human β2 subunit tagged with YFP at its C-terminus, C-terminal KPI residues deleted
SEQ ID NO: 79	SEQ ID NO: 80	Rat γ1 subunit tagged with YFP at its C-terminus, C-terminal LTGGEKKP residues deleted
SEQ ID NO: 81	SEQ ID NO: 82	Rat α2 subunit tagged with mseCFPΔ11 at its C-terminus, C-terminal AR residues deleted
SEQ ID NO: 76	SEQ ID NO: 85	Rat α2 subunit-EEEEKKKK helix at its C-terminus, C-terminal AR residues deleted, tagged with eCFP with MVSK N-terminal

		residues deleted
SEQ ID NO: 83	SEQ ID NO: 84	Rat α2 subunit EEEEKKKK helix at its C-terminus, C-terminal AR residues deleted, tagged with mseCFPΔ11 with MVSK N-terminal residues deleted
SEQ ID NO: 86	SEQ ID NO: 87	Human β2 subunit, pMDK vector
SEQ ID NO: 88	SEQ ID NO: 89	Human β2 subunit tagged with cpVenus at its C-terminus, C-terminal KPI residues deleted
SEQ ID NO: 90	SEQ ID NO: 91	Rat γ1 subunit, pMDS vector
SEQ ID NO: 92	SEQ ID NO: 93	Rat γ1 subunit tagged with cpVenus at its C-terminus, C-terminal LTGGEKKP residues deleted
SEQ ID NO: 94	SEQ ID NO: 95	Rat γ1 subunit, valine 275 and leucine 276 replaced by glycine residues V275G+L276G. Negative control
SEQ ID NO: 96	SEQ ID NO: 97	Human β1 subunit tagged with YFP at its C-terminus, C-terminal KPI residues deleted
SEQ ID NO: 98	SEQ ID NO: 99	Human β1 subunit

The present invention further concerns the following embodiments:

(a) A heterotrimeric AMP-activated protein Kinase (AMPK) comprising a
5 fluorescent dye pair allowing detection and/or measurement of conformational changes within the AMPK complex.

(b) A heterotrimeric AMP-activated protein Kinase (AMPK) comprising a fluorescent dye pair allowing detection and/or measurement of conformational
10 changes within the AMPK complex, allowing the detection and/or measurement of allosteric AMPK activation

(c) The heterotrimeric AMPK as defined in embodiments (a) or (b), wherein the fluorescent dyes are chosen among Foerster transfer pairs, more particularly
15 genetically encoded fluorescent proteins such as GFP and GFP derived proteins such as CFP/YFP, $mseCFP_{\Delta 11}$/cpVenus, or constructs thereof.

(d) A trimeric AMPK as defined in embodiments (a), (b) or (c) characterized in that it comprises an α-subunit, that is α1or α2, a β-subunit that is either β1 or β2, and γ-subunit that is either γ1 or γ2 or γ3, or fragments thereof, two of the subunits being tagged with one of the fluorescent dyes.

(e) An AMPK as defined in embodiments (a), (b), (c) or (d) comprising α2, β2 and γ1 subunits, wherein the α2 subunit is tagged with CFP or $mseCFP_{\Delta 11}$ at the C-terminus and the β2 or the γ1 subunit is tagged with YFP or cpVenus at the C-terminus.

(f) A nucleic acid molecule encoding the trimeric AMPK as defined in embodiments (a) to (e).

(g) A vector comprising the nucleic acid molecule of embodiment (f).

(h) The vector of embodiment (h) which is an expression vector wherein the nucleic acid molecule of embodiment (h) is operatively linked to one or more control sequences allowing the expression in prokaryotic and/or eukaryotic hosts.

(i) A host containing at least one vector as defined in embodiments (g) or (h) or at least one nucleic acid molecule as defined in embodiment (f), the host being preferably a bacteria, an insect, fungal, plant or animal cell such as a mammalian cell and more preferably a human cell or human cell line.

(j) A method for identifying an AMPK allosteric interactor and/or its concentration in a sample comprising contacting the sample with AMPK as defined in anyone of embodiments (a) to (e) or a host cell as defined in embodiment (i) and detecting a modification of the fluorescence by fluorescent techniques such as FRET.

(k) An *in vivo* method of screening an AMPK allosteric interactor, the method comprising:

- providing a cell culture comprising cells expressing the trimeric AMPK as defined in anyone of embodiments (a) to (e);
- providing candidate allosteric interactors;
- contacting the cells with said candidate allosteric interactor; and

- detecting a modification of the fluorescence by fluorescent techniques such as FRET.

(l) Use of FRET signal to detect a conformational change of the AMPK as defined in anyone of embodiments (a) to (e).

(m) Use of FRET signal as defined in embodiment (l) to identify an allosteric interactor of the AMPK as defined in anyone of embodiments (a) to (e).

(n) Use of the AMPK as defined in anyone of embodiments (a) to (e) to quantify changes in cellular AMP and ADP levels.

(o) A kit for identifying the presence of an allosteric interactor of AMPK in a sample, the kit comprising a trimeric AMPK as defined in anyone of embodiments (a) to (e), the reagents and instructions for use.

Brief description of the figures

Figure 1: **Conformational change model showing operating mode of AMPfret sensors.**

AMPfret sensors are constructed from an AMPK heterotrimer (consisting of α-, β-, and γ–subunits) with two additional GFP-derived fluorescent proteins (CFP, YFP) fused to different N- and C-termini of AMPK subunits. Binding of AMP or ADP to two CBS domains in the AMPK γ-subunit induces a conformational change which reduces the distance between the fluorophore couple. This increases fluorescence (or Foerster) resonance energy transfer (FRET) between the two fluorophores. Experimentally, when CFP is excited at 439 nm, FRET reduces direct CFP fluorescence emission at 476 nm, while energy transferred to YFP increases YFP fluorescence emission at 527 nm.

Figure 2: **Initial AMPfret constructs.**

AMPfret A and C exhibit variation of FRET ratio upon AMP binding. Top: Schema showing structural organization of the sensors. CFP and YFP are respectively represented as hatched- and dotted-circles. **(a)** Fluorescence emission spectra of AMPfret constructs excited at 430 nm. Spectra show fluorescence peaks of CFP (476 nm) and YFP (527 nm), and their variation upon AMP binding (dotted line: 3 mM ATP,

continuous line: 20 µM AMP). **(b)** FRET variation of AMPfret constructs calculated from data above (hatched column: 3 mM ATP, dotted column: 20 µM AMP) and autoradiograms of *in vitro* kinase activity assays with these constructs using acetyl-CoA carboxylase (ACC) as a substrate. Data correspond to mean ± SEM (AMPfret A: 5 n=7; AMPfret C: n=10). Note: AMPfret constructs exhibit similar activity as native AMPK.

Figure 3: Optimized AMPfret constructs.

Second generation of AMPfret constructs 1.1 and 2.1. based on constructs 10 AMPfret C and A, respectively. Top: Schema showing structural organization of the sensors. Both optimized constructs contain mseCFP$_{\Delta11}$/cpVenus as GFP-derived fluorescent couple instead of CFP/YFP. mseCFP$_{\Delta11}$ and cpVenus are respectively represented as hatched- and checkered circles. AMPfret 2.1 α- and β-subunits also contain small deletions in their protein sequence to shorten C-terminal non-folded 15 linker sequences (A_{551} and R_{552} in α and K_{270}, P_{271} and I_{272} in β). In addition, a putatively rigid helix (7 amino acids) was inserted between the α-subunit C-terminus and CFP (see small box with curled lines). **(a)** Fluorescence emission spectra of AMPfret constructs excited at 430 nm. Spectra show fluorescence peaks of mseCFP$_{\Delta11}$ (476 nm) and cpVenus (527 nm), and their variation upon AMP binding (dotted line: no AMP, 20 continuous line: 20 µM AMP). **(b)** FRET variation of AMPfret constructs calculated from data above (hatched column: no AMP, dotted column: 20 µM AMP) and autoradiograms of *in vitro* kinase activity assays with these constructs using acetyl-CoA carboxylase (ACC) as a substrate. Data correspond to mean ± SEM (AMPfret 1.0: n=10; AMPfret 1.1: n=7); *= p < 0,001 (significance assessed by a Student-Newman-25 Keuls test). Note: All AMPfret constructs exhibit similar activity as native AMPK. AMPfret 2.1 reveals improved FRET variation range as compared to AMPfret 1.1, providing proof of principle that optimization of FRET is possible.

Figure 4: FRET response of AMPfret sensors correlates with the concentration 30 of AMPK activator AMP.

AMP concentration dependence of the normalized FRET ratio of AMPfret sensors **(a)** AMPfret 1.1 and **(b)** AMPfret 2.1. The FRET ratio was calculated from fluorescence emission spectra excited at 430 nm. Data points correspond to mean ± SEM (n≥3). Data were fitted with Sigma Plot 1.1 software to single site binding 35 kinetics, yielding affinities of 1,8 µM (AMPfret 1.1) and 1,5 µM (AMPfret 2.1.). Note:

AMPfret sensors are sensitive to AMP concentrations in a physiological range (0-20 µM)

Figure 5: FRET response of AMPfret sensors correlates with the concentration of AMPK activator ADP.

ADP concentration dependence of the normalized FRET ratio of AMPfret sensors **(a)** AMPfret 1.1 and **(b)** AMPfret 2.1. The FRET ratio was calculated from fluorescence emission spectra excited at 430 nm. Data points correspond to mean ± SEM (n≥3). Data were fitted with Sigma Plot 1.1 software to single site binding kinetics, yielding affinities of 5 µM (AMPfret 1.1) and 7,4 µM (AMPfret 2.1). Note: AMPfret sensors are sensitive to ADP concentrations in a physiological range (0-50 µM for free ADP).

Figure 6: AMPfret sensors as *in vitro* tools to identify AMPK allosteric activators.

AMPfret 2.1 is incubated in absence (grey mesh bars) or in presence (black bars) of **(a)** 20 µM AMP, **(b)** 50 µM A-769662 or **(c)** 500 µM Metformin. Structure and names of the molecules are given below the bars. Data correspond to mean ± SEM (AMP: n=7; A-769662: n=4; Metformin: n=4); *= p < 0,001 (significance assessed by paired T-test).

Figure 7: AMPfret sensors as cellular *in vivo* tools to identify AMPK allosteric activators – HeLa cells

HeLa cells transfected with AMPFret 2.1 were exposed for 60 min to 1 mM AICAR. **(a)** Fluorescence emission spectra showing the increase of cpVenus peak (527nm) over time; dotted black line: 0 min; dashed black line: 15 min and solid black line: 30 min. **(b)** Time course of the FRET signal. Normalized FRET ratio determined each 15 minutes (mean ± SEM; n=45; *= p < 0,001 according to the performed Mann-Whitney Rank Sum Test). **(c)** AMPK activation at t=0 min and t=60 min. Phosphorylation of the AMPK substrate ACC as determined by immunoblotting (lower panel) and quantification of the resulting P-ACC/total ACC ratio (upper panel). P-ACC/total ACC ratios at t=0 and t=60min are respectively represented as a white dotted bar and black dotted bar. Data correspond to mean ± SEM (n=3).

Figure 8: AMPfret sensors as cellular *in vivo* tools to identify AMPK allosteric activators – 3T3-L1 cells

HeLa cells transfected with AMPFret 2.1 were exposed for 60 min to 1 mM AICAR. **(a)** Time course of the FRET signal. Normalized FRET ratio determined each 15 minutes (mean ± SEM; n=9). **(b)** Time course of AMPK activation. Phosphorylation of the AMPK substrate ACC as determined by immunoblotting (lower panel) and 5 quantification of the resulting P-ACC/total ACC ratio (upper panel). Data correspond to mean ± SEM (n=3).

Figure 9: Effect of 1 hour ischemia followed by 1 hour reperfusion on HepG2 cell followed by AMPfret.

10 AMPfret 2.1 normalized FRET ratio evolution during 1h ischemia (light grey bar) and 1h reperfusion (dark grey bar). Transfected HepG2 cells were cultured on a glass slide mountable onto the incubation flow-through chamber of our Leica TCS SP2 AOBS confocal microscope. At t=0, the cell was placed under ischemia-like conditions: hypoxic conditions (2% O2) and glucose-free medium at 37°C. Deprived medium was 15 previously bubbled with N2 for at least 10 minutes before its addition onto the cells. After 1hour of deprivation, started the 1 hour-reperfusion period with glucose-rich medium and O2 (21%). FRET values were record every minute from a single isolated cell using the Leica confocal software. The FRET ratio was followed by recording simultaneously mseCFPΔ11 (476 nm) and cpVenus (527 nm) fluorescence emitted 20 within 4 nm windows using two independent channels, under excitation set at 458 nm. FRET ratio was normalized to 1 at t=0.

Figure 10: Strategy for optimizing the AMPK sensors according to the present invention. Starting from the most promising original constructs, FRET signal was 25 optimized by mutations, deletions and addition of sequences.

The compounds and processes of the present invention will be better understood in connection with the following examples, which are intended as an illustration only and not limiting of the scope of the invention

30

Example 1: AMPK constructs

AMPK constructs and protein preparation

The α_2, β_2 and γ_1 AMPK subunits tagged or not with fluorescent protein, were 35 respectively cloned in the pACE, pDC and pDS vectors of the ACEMBL expression system (Bieniossek et al., 2009, Automated unrestricted multigene recombineering for multiprotein complex production, Nat Meth 6(6):447-50) using SLIC (Li et al., Methods

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Mol Biol 852:51, 2012) and conventional cloning. Created vectors, containing a single subunit fluorescently tagged or not, were fused via their Lox-P site using the CRE-recombinase (EMBL Heidelberg): a single expression vector coding for a chimeric AMPK that contains two of its three subunits flanked with the mseCFP$_{\Delta11}$/cpVenus fluorescent proteins pair (respectively variant of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) at their termini was obtained. A decaHis-tag, cleavable by the TEV protease, was inserted at the N-terminus of the α_2 subunit in order to purify easily the heterotrimer.

BL21 (DE3) *Star* cells were transformed by electroporation and protein expression was carried overnight at 18°C in autoinducing medium. Cells were collected by centrifugation at 6000 rpm for 20 min using a Beckman Coulter centrifuge (rotor JLA-8.1000) and wash with PBS. Cells were then suspended in lysis buffer (0,5 M sucrose, 30% glycerol, 50 mM Tris pH8, 100 mM NaCl, 2 mM MgCl2, 2 mM β-mercaptoethanol, lysosyme 1 mg/mL, 20 mM imidazole, Complete EDTA free tablet (Roche), leupeptin, pepstatin). 200 U Benzonase were added to the suspension, and it was gently stirred for 1 h in the cold room. Cells were then lysed by sonication using a MisonixSonicator 4000 (5 min total at 80% - 20 s ON / 1 min OFF).

Cell-free extract, obtained by centrifugation at 20'000 rpm for 80 min (rotor JS 25.50) was applied on Ni-NTA Superflow resin (Qiagen) pre-equilibrated with lysis buffer. Resin was washed using washing buffer (50 mM Tris pH 8, 100 mM NaCl, 20 mM imidazole, 2 mM MgCl2, 2 mM β-mercaptoethanol) and high salt buffer (wash buffer + 1 M NaCl). Proteins were eluted by applying elution buffer (wash buffer + 400 mM Imidazole). Imidazole was removed through an overnight dialysis in buffer A (50 mM Tris pH8, 100 mM NaCl, 2 mM MgCl2, 2 mM β-mercaptoethanol). Eluted proteins were passed over a 5 mL QXL column (GE Healthcare) in order to remove proteins bound to nucleic acids and non-stoichiometric AMPK complexes. Proteins were eluted using a gradient of buffer B (50 mM Tris pH8, 1 M NaCl, 2 mM MgCl2, 2 mM β-mercaptoethanol). Finally chimeric AMPK heterotrimers were applied to a Superose™ 6 gel filtration column (GE Healthcare) pre-equilibrated with SEC buffer (50 mM Tris pH8, 200 mM NaCl, 2 mM MgCl2, 2 mM β-mercaptoethanol, 5 mM spermidine). Spermidine diminished concentration dependent AMPK oligomer formation. After adding glycerol to a final concentration of 50%, the purified AMPK (untagged or AMPK 221WT) and AMPK heterotrimers of the invention (AMPK tagged hereafter AMPFret or AMPFret sensors) were stored at -20°C for further experiments.

Finally, combinations of AMPK tagged with mseCFP$_{\Delta11}$ and cpVenus on two of the termini of its 3 subunits were created in order to identify constructs that show FRET signal variation upon AMP binding (hereafter termed AMPFret).

Table 1 – Overview of the AMPfret constructs containing two fluorescent protein tags permutated at the N- and C-termini of the three AMPK subunits.

AMPfret construct	Vector name and composition
AMPK 221	pACEMBL α_2 _ β_2 _ γ_1
AMPfret A	pACEMBL α_2-CFP _ β_2-YFP _ γ_1
AMPfret C	pACEMBL α_2-CFP _ β_2 _ γ_1- YFP

5

Abbreviations: pACEMBL, plasmid resulting from the Cre-LoxP fusion of vectors pACE, pDC and pDS of the MutliColi expression system; CFP, Cyan Fluorescent Protein; YFP, Yellow Fluorescent Protein; α2, β2, γ1, AMPK subunits.

10

Characterization of AMPFret sensors *in vitro*

ATP containing buffers were always freshly prepared to limit AMP contamination. Aqueous solutions of nucleotides (adenine nucleotides, NAD) were analyzed by HPLC (stationary phase: Polaris C18 / mobile phase: 60% CH_3CN 40% H_20)
15 to evaluate spontaneous ATP and ADP hydrolysis and contaminations.

Enzymatic assay: AMPK 221WT and AMPfret constructs (3 pmol) were activated by incubation with purified CamKKβ (1 pmol) for 20 min at 30°C in kinase buffer (200 µM ATP, 40 µM AMP, 5 mM MgCl2, 1 mM DTT and 10 mM Hepes pH 7.4). Purified ACC fragment targeted by AMPK (200 pmol) was then incubated for 20 min at 37°C in
20 presence or absence of pre-activated AMPK 221WT or AMPFret sensor in kinase buffer containing [γ-^{32}P]ATP. Reaction mixtures were then load on SDS-PAGE gel, P-ACC signals were revealed using a Typhoon™ and activities were evaluated with ImageJ.

FRET assay: FRET signal variation in presence of different compounds (nucleotides, chemicals, ions) was measured using a fluorimeter (Photon Technology
25 International). AMPfret constructs (20 pmol) were incubated in a quartz cuvette in a final volume of 150 µL (spectro buffer: 50 mM Tris pH8, 200 mM NaCl, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol). Effects of nucleotides and others compounds (previously

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prepared in the spectro buffer) on the FRET ratio given by AMPfret sensor was determined by comparing FRET ratio (peak value at 527 nm / peak value at 476 nm) in presence or absence of the compounds. Excitation wavelength was set to 430nm, and emission spectra were recorded from 450 to 600 nm with an integration time of 0,2 s.
5 Mg^{2+} effect on FRET was investigated in spectro buffer without Mg^{2+}.

The two constructs, AMPFret A (α_2-CFP-β_2-YFP-γ_1; CFP tagged at the α2 C-terminus, and YFP at the β1 C-terminus) and AMPFret C (α_2-CFP-β_2-γ_1-YFP; CFP tagged at the α2 C-terminus, and YFP at the γ1 C-terminus) showed both a significant difference in their FRET signal (~10%) depending on the presence of AMP or ADP
10 (Figure 2). It appears that, during allosteric activation, the α-subunit C-terminus approaches the C-termini of the β- and γ-subunits.

Example 2: Optimized AMPK constructs

15 Constructs were optimized to achieve a superior FRET signal amplitude. The construct AMPfret 1.1 is based on AMPfret C, containing full length AMPK subunits α2, β2 and γ1. The α-subunit is tagged with mseCFPΔ11 at its C-terminus and the γ-subunit is tagged at its C- terminus with cpVenus; the β-subunit remains untagged. The construct AMPfret 2.1 is based on AMPfret A, where CFP/YFP were exchanged for
20 the same different fluorophore pair as AMPfret 1.1. In addition, the sequence of the construct was modified. First, small truncations based on the crystal structure (PDB 2Y94) and secondary structure prediction (nps@consensus (ucbl)) were inserted via PCR and "self SLIC" between the N-terminus of fluorescent protein tags and the C-terminus of the tagged AMPK subunits. Such shortening of the sequence between
25 AMPK core and tag may remove flexibility other than the conformational change induced by AMP. Second, a short insert supposed to fold into a rigid α-helix (Sivaramakrishnan et al., PNAS 105:13356, 2008 and 108:20467, 2011) was inserted between the α2 C-terminus and the CFP N-terminus to rigidify the AMPK backbone of the invention and to stabilize the CFP tag in a given position relative to AMPK.
30 This engineering comprised the following mutations. The last 2 C-terminal amino acids (AR) of α2 and the first 3 N-terminal amino acids (MSK) of mseCFPΔ11 were removed and the new termini linked via 8 amino acids insert supposed to fold into an α-helix (EEEEKKKK, SEQ ID No.1). Further, the last C-terminal (non-folded) 3 amino acids (KPI) of β2 were also removed, and directly fused to the N-terminus of YFP. Since
35 2 amino acids resulting from the restriction site previously used were also removed by the SLIC technique, this yielded a construct lacking in total 5 amino acids between the

β2-subunit and YFP. The optimized AMPfret sensor showed an almost 100% increased FRET ratio (Figure 3).

The optimized AMPfret sensors allow titration of the allosteric AMPK-activators AMP and ADP, confirming that both induce conformational changes in the AMPK
5 heterotrimer. The affinity (Kd) for AMP and ADP could be determined as 1,5 µM and 7,4 µM, respectively.

The AMPfret sensors thus represent a pioneering powerful and easy-to-use tool to decipher the activation mechanisms of AMPK. They contain full length AMPK heterotrimer that behaves the same way as native AMPK WT as judged by (i) its kinase
10 activity after phosphorylation via CamKKβ and allosteric activation by AMP and (ii) its affinities for adenine nucleotides. The AMPfret FRET signal is directly dependent of the AMP concentration; in a physiological range (1 – 10µM) it shows almost linear relationship (Figures 4 and 5).

15 **<u>Example 3:</u> *in vitro* interaction of optimized AMPfret with allosteric activators**

The optimized AMPfret sensors not only translate the adenylate-dependent movements of the AMPK heterotrimer into a FRET signal, which are triggered by adenylate binding to specific sites at the γ-subunit. Their readout also reports
20 conformational changes of other, pharmacological direct AMPK activators such as the compound A-769662 (Figure 6). This molecule interacts with the β-subunit, but clearly induces a FRET signal comparable to AMP, even if the triggering conformational change may be of different nature according the different binding mode.

Metformin, a widely used anti-diabetes drug, which was postulated to directly
25 interact with γ-subunit (Zhang et al., Mol Cell Biochem 368:69, 2012) did not induce any FRET variation emission of AMPfret (Figure 6). This absence of conformational changes *in vitro* supports the generally accepted indirect mode of action, whereby this drug inhibits mitochondrial respiration and increases the AMP/ATP ratio, thus activating AMPK by the canonical AMP binding mechanism at the γ-subunit.
30 Taken together, AMPfret appears as a valuable and accurate tool for *in vitro* applications, notably screening for AMPK interactors.

<u>Example 4:</u> *Ex vivo* experiments with the optimized AMPfret constructs

35 For cellular ***ex vivo*** experiments, subunits of the optimized construct AMPfret 2.1 were cloned in the vectors (pACEMam2, pMDS and pMDK) of the

*Multi*Mam expression system. Created plasmids were fused via their Lox-P site to yield to a single mammalian expression vector coding for the sensor AMPFret 2.1 according to well-known techniques to the skilled man in the art.

3T3-L1 and HeLa cells were cultivated in glucose containing DMEM (4,5 g/L) supplemented with SVF, glutamine, penicillin and streptomycin. Once cells reached around 80% confluence, medium was replaced by Opti-MEM™ (Lifetechnologies) and AMPfret 2.1 coding plasmid was transfected using Lipofectamine2000 (Lifetechnologies). After 5h, Opti-MEM™ was exchanged by complete DMEM and cells grew for >36h until their observations under the confocal microscope.

3T3-L1 or HeLa cells, cultivated in 8 wells LabTek cover glass plates (Nunc), were observed with a Leica TCS SP2 AOBS confocal microscope. LabTek plates were placed in an incubation chamber in which the temperature and O_2 concentration were maintained at 37°C and 21%, respectively. Without moving the Labtek, 200 µL medium was replaced by the same volume of complete medium containing 2 mM AICAR (1 mM final). Excitation wavelength was set to 458 nm and emission spectra showing FRET signal were monitored through λ scans from 463 nm to 600 nm every 15 min. ROI (region of interest) were drawn in order to cover entire cells. FRET ratio variations were calculated from those measured emission spectra.

Under the microscope, cells were treated with 1 mM AICAR (AMPK allosteric activator) to visualize the allosteric activation of AMPK through the FRET signal of AMPfret 2.1 and hence validate its use for ***ex vivo*** applications.

AMPfret 2.1 was excited using a 458 nm laser and emission spectra showing FRET signal were monitored through λ scans from 463 nm to 600 nm every 15 min. The AMPfret 2.1 FRET signal increased with time upon AICAR addition, suggesting that AMPfret 2.1 can monitor allosteric activation of AMPK in cells (Figures 7 and 8).

More than half-maximal response was already reached after 15 min of treatment, and the maximal effect reached after about 30 minutes.

The activation kinetics of AMPK upon AICAR addition was independently verified by Western blotting for the AMPK-specific phosphosite in acetyl-CoA carboxylase (ACC; widely used as reporter for AMPK activity) in 3T3-L1 cells.

All the results presented above show that the AMPfret sensor can be used as a suitable tool for cellular *in vivo* applications.

<u>Example 5:</u> AMPfret 2.1 during ischemia-reperfusion in a HepG2 single cell

Using an incubation flow-through chamber fitted to the confocal microscope which permits to control temperature as well as O2 concentration, HepG2 cells were

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placed under ischemia-like conditions, comprising hypoxic conditions (2% O2) and glucose free medium at 37°C. ATP pools may not be affected when hypoxia is applied in a high nutrient containing medium since cells can adapt to hypoxia by switching their energy metabolism through anaerobic pathways to compensate for aerobic ATP
5 production. The deprivation period was followed by 1 hour of reperfusion with complete medium and O_2 (21%). During the 2 hours of the ischemia-reperfusion protocol, the FRET ratio was monitored every minute by recording simultaneously mseCFPΔ11 and cpVenus fluorescence emitted within 4 nm windows (corresponding to fluorescence emission) using two independent channels. Images were collected and
10 processed using ImageJ in order to i) remove eventual background fluorescence and ii) isolate individual cells from acquired pictures. Then, the fluorescence intensities were extracted from single cell images using Volocity™. Thus, the effect of ischemia-reperfusion on the AMPfret 2.1 signal in single cells was analyzed (Figure 9).

15 During ischemia in HepG2 cells, the FRET signal did not vary. Changes in AMP/ATP ratio under such conditions were proposed to happen in the liver and AMPK becomes activated, but a recent study suggested that AMPK was activated during ischemia through adenylate-independent pathways. Figure 9 shows results of a single cell.

20 During reperfusion of HepG2 cells, the FRET signal increases over the first 30 minutes indicating increased AMP and ADP concentrations. Subsequently, the FRET ratio remained at unchanged high values, suggesting that elevated AMP and ADP concentrations were maintained. In fact, AMPfret should revert the FRET ratio as soon as AMP and ADP levels drop again. These results suggest that in HepG2 cells,
25 reperfusion represented a more drastic energy stress than ischemia regarding adenylate concentrations and AMPK allosteric activation.

Through these experiments, using AMPfret 2.1 in HepG2 cells, we did not detect any FRET signal changes during ischemia, suggesting that AMP and ADP concentrations
30 remained unchanged. However, we showed an increase of AMPfret FRET signal during reperfusion, suggesting an elevation of intracellular AMP and ADP and allosteric activation of AMPK.

These experiments achieved in living cells using AMPfret 2.1 show that AMPfret 2.1 was properly transfected and its fluorescence monitored over time. These results

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show that AMPfret 2.1 provides a readout of AMP/ZMP concentrations and AMPK allosteric activation by reporting the related conformational changes. Experiments involving ischemia-reperfusion showed that AMPfret 2.1 can monitor endogenous changes of adenylates and AMPK allosteric activation over time.

5

Monitoring of transient events related to AMPK allosteric activation is promising to decipher or unravel new aspects of its regulation.

CLAIMS

1. A heterotrimeric mammalian AMP-activated protein kinase (AMPK) construct consisting in (i) an α-subunit of AMPK or a mutant or a fragment thereof, (ii) a β-subunit of AMPK or a mutant or a fragment thereof, (iii) a γ-subunit of AMPK or a mutant or a fragment thereof, wherein the kinase activity and the regulatory domains of AMPK in said mutants and fragments are retained such that allosteric activation of AMPK is allowed, and (iv) a fluorescent dye pair tagging at least two of (i) to (iii), said fluorescent dye pair being placed to allow detection of conformational changes within the AMPK construct.

2. The AMPK construct of claim 1, wherein the heterotrimer is constitutively stable.

3. The AMPK construct of claim 1 or 2, wherein (i) is AMPK α1, AMPK α2 or a mutant or a fragment of AMPK α1 or AMPK α2, (ii) is AMPK β1, AMPK β2 or a mutant or a fragment of AMPK β1 or AMPK β2 and (iii) is AMPK γ1, AMPK γ2, AMPK γ3 or a mutant or a fragment of AMPK γ1, AMPK γ2 or AMPK γ3,

wherein the kinase activity and the regulatory domains of AMPK in said mutants and fragments are retained such that allosteric activation of AMPK is allowed.

4. The AMPK construct of any one of claims 1 to 3, wherein two of (i) to (iii) are tagged with the fluorescent dye pair.

5. The AMPK construct of any one of claims 1 to 4, wherein (i) and either (ii) or (iii) are tagged.

6. The AMPK construct of any one of claims 1 to 5, wherein (i) is tagged at the C-terminus.

7. The AMPK construct of any one of claims 1 to 6, wherein either (ii) or (iii) is tagged at the C-terminus.

8. The AMPK construct of any one of claims 1 to 5, wherein (i) is tagged at the C-terminus and either (ii) or (iii) is tagged at the C-terminus.

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9. The AMPK construct of any one of claims 1 to 8, consisting in α2, β2 and γ1 subunits.

10. The AMPK construct of claim 9, wherein the α2 subunit is tagged at the C-terminus and either the β2 or the γ1 subunit is tagged at the C-terminus.

11. The AMPK construct of any one of claims 1 to 10, wherein the fluorescent dye pair is a Förster or fluorescence resonance energy transfer (FRET) pair.

12. The AMPK construct of any one of claims 1 to 10, wherein the fluorescent dye pair consists in genetically encoded fluorescent proteins.

13. The AMPK construct of claim 12, wherein the fluorescent dye pair consists in GFP or a GFP derived protein, or constructs thereof.

14. The AMPK construct of claim 13, wherein the fluorescent dye pair is CFP / YFP or mseCFP$_{\Delta 11}$ / cpVenus.

15. The AMPK construct of any one of claims 1 to 14, consisting in α2, β2 and γ1 subunits, wherein the α2 subunit is tagged with mseCFPΔ11 at the C-terminus and either the β2 or the γ1 subunit is tagged with cpVenus at the C-terminus or wherein the α2 subunit is tagged with CFP at the C-terminus and either the β2 or the γ1 subunit is tagged with YFP at the C-terminus.

16. The AMPK construct of any one of claims 1 to 15, wherein said mammal is murine, simian, equine, human, bovine or ovine.

17. The AMPK construct of claim 16, wherein the α, β and γ subunits originate from mice, rat, human, bovine or ovine or any combination thereof.

18. A nucleic acid molecule encoding the heterotrimeric mammalian AMP-activated protein kinase (AMPK) construct defined in claim 1.

19. A vector comprising the nucleic acid molecule defined in claim 18.

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20. The vector of claim 19, which is an expression vector wherein the nucleic acid molecule defined in claim 18 is operatively linked to one or more control sequences allowing expression of said nucleic acid molecule in prokaryotic and/or eukaryotic hosts.

21. A host cell containing at least the vector defined in claim 19 or 20 or at least the nucleic acid molecule defined in claim 18.

22. The host cell of claim 21, which is a bacterium, insect, fungus, plant or animal cell.

23. The host cell of claim 22, which is a mammalian cell.

24. The host cell of claim 23, which is a human cell or human cell line.

25. A method for identifying an AMPK allosteric interactor and/or its concentration in a sample comprising the steps of:

a. contacting the sample with the heterotrimeric mammalian AMPK construct defined in any one of claims 1 to 17 or the host cell defined in any one of claims 21 to 24, and

b. detecting a modification of fluorescence resonance energy transfer (FRET) fluorescence by fluorescence techniques.

26. An *in vitro* method of screening an AMPK allosteric interactor, the method comprising:

- providing a cell culture comprising cells expressing the heterotrimeric mammalian AMPK construct defined in any one of claims 1 to 17;
- providing candidate allosteric interactor;
- contacting the cells with said candidate allosteric interactor; and
- detecting a modification of fluorescence resonance energy transfer (FRET) fluorescence by fluorescent techniques.

27. Use of the mammalian AMPK construct defined in any one of claims 1 to 17, for the detection of conformational changes within AMPK.

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28. Use of the mammalian AMPK construct defined in any one of claims 1 to 17, for the identification of an allosteric interactor of AMPK.

29. The use according to claim 27 or 28, wherein the detection of conformational changes within AMPK or the identification of the interactor of AMPK comprises measuring change in fluorescence resonance energy transfer or in fluorescence quenching.

30. Use of the mammalian AMPK construct defined in any one of claims 1 to 17, to quantify changes in cellular AMP and ADP levels.

31. A kit for identifying the presence of an allosteric interactor of AMPK in a sample, the kit comprising the mammalian AMPK construct defined in any one of claims 1 to 17, reagents and instructions for use.

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FIG. 1

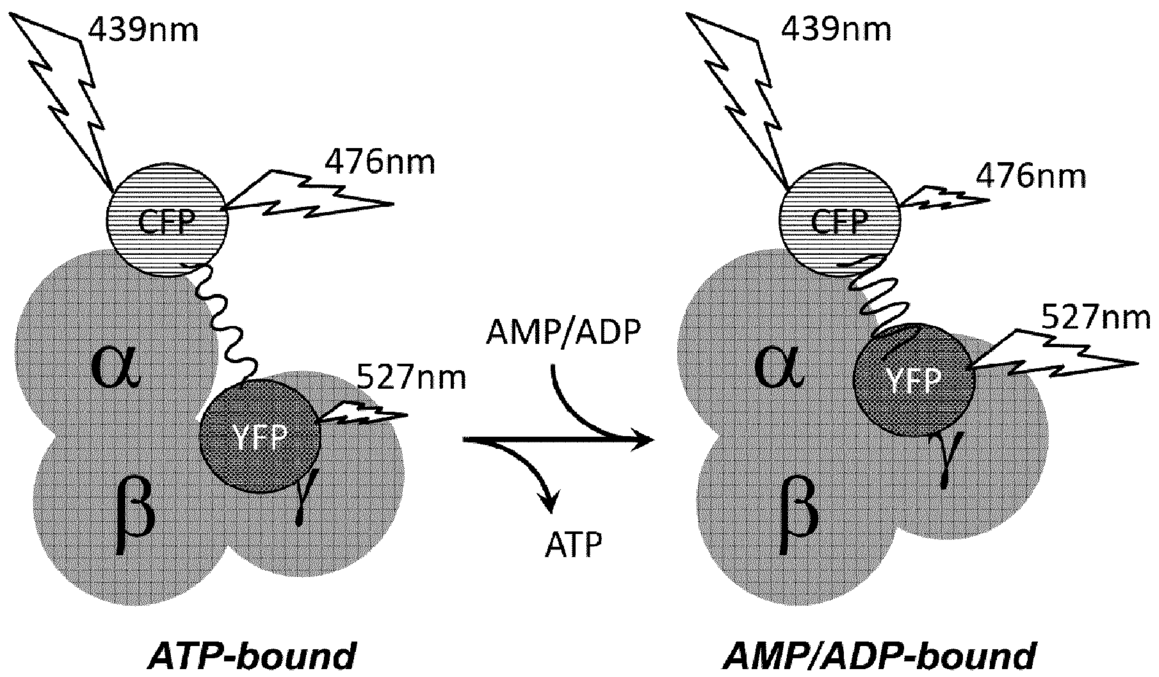


FIG. 2

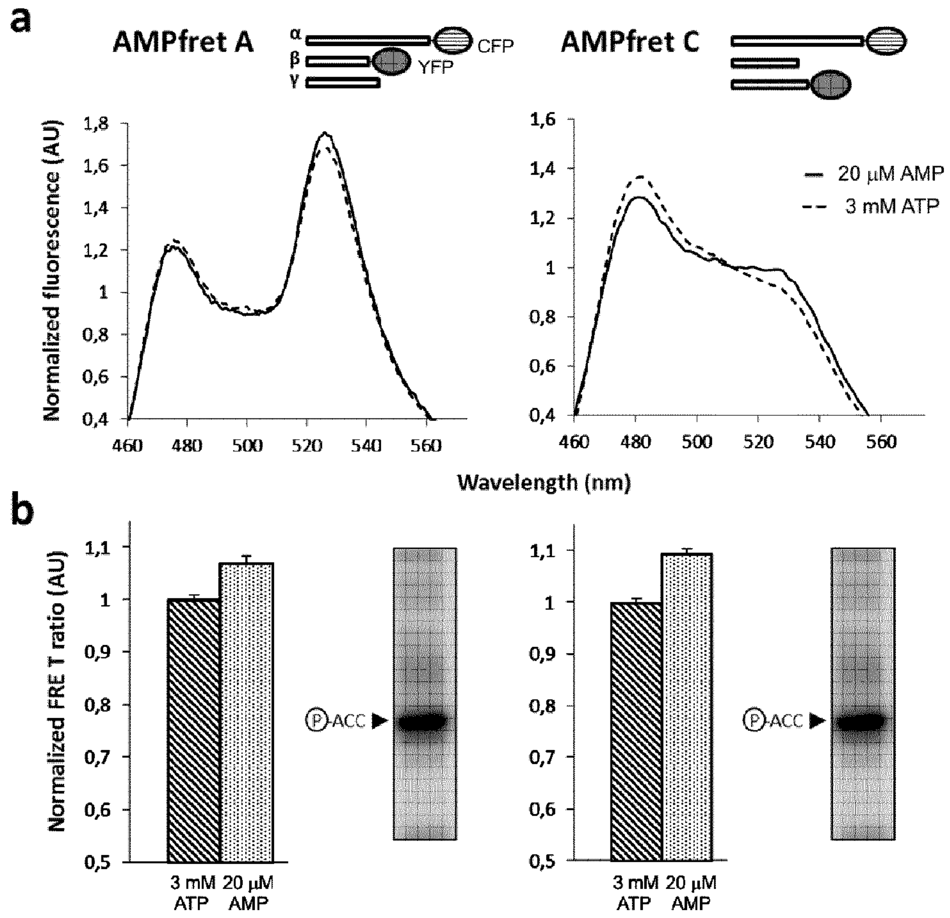


FIG. 3

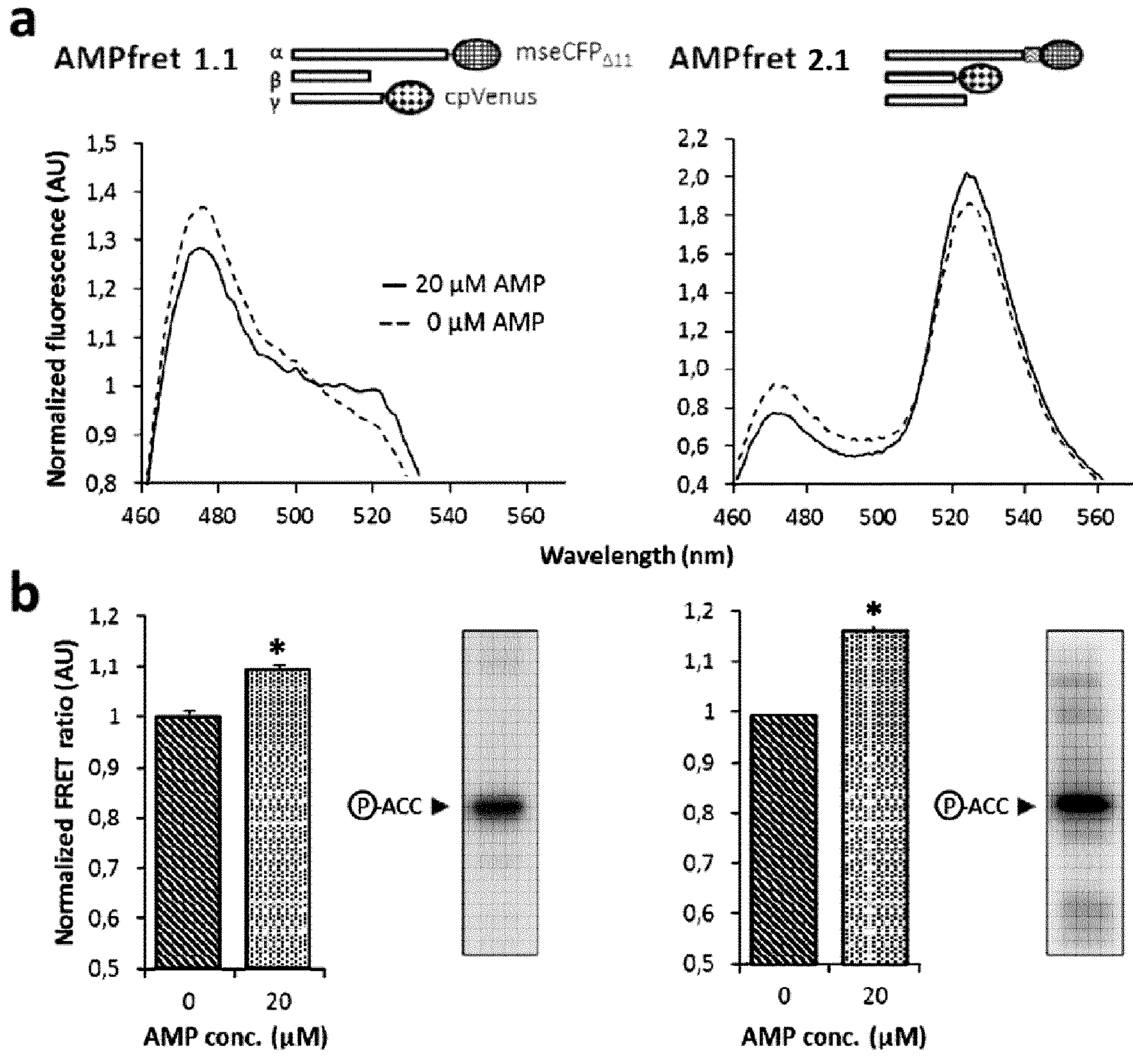


FIG. 4

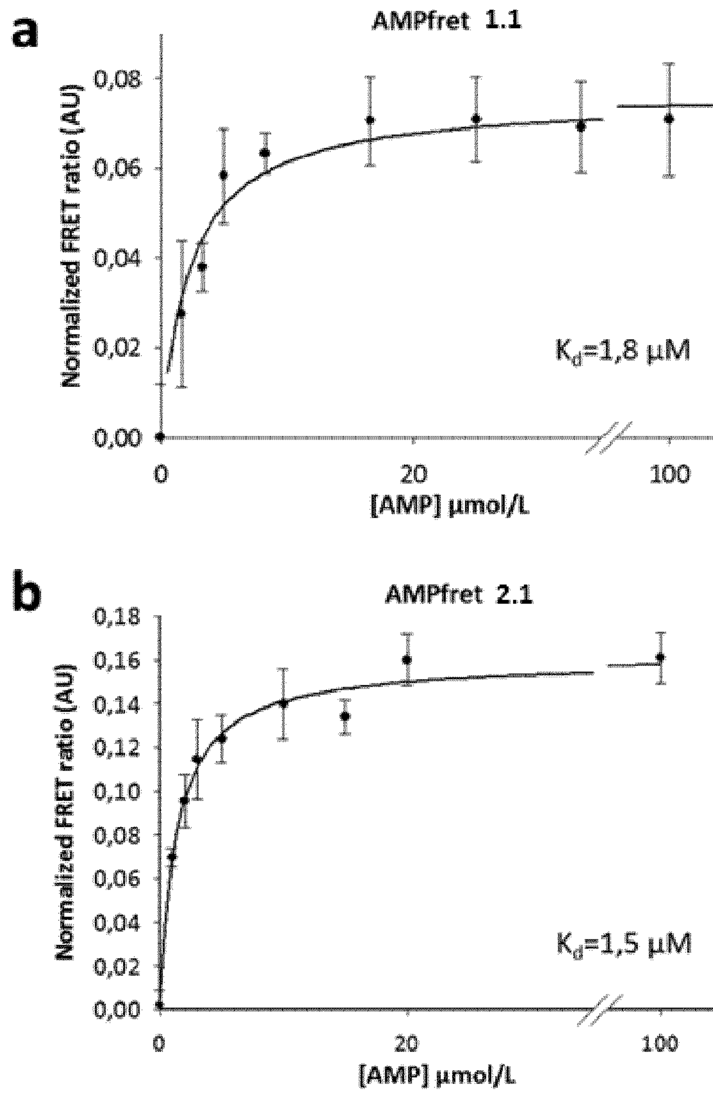


FIG. 5

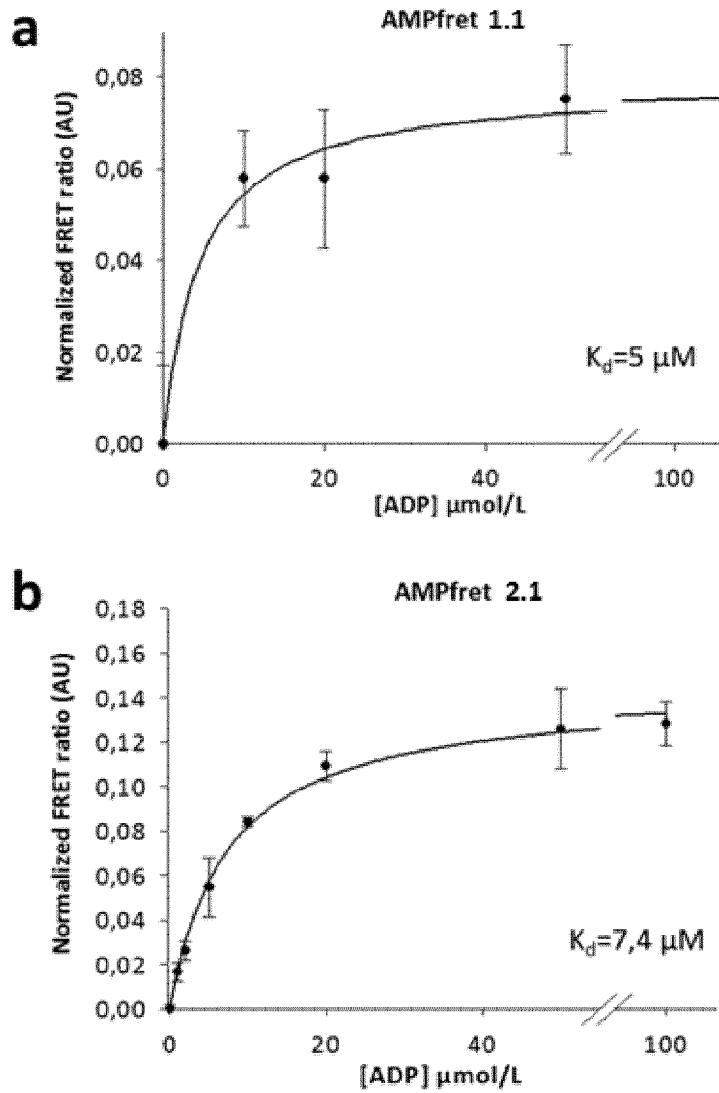


FIG. 6

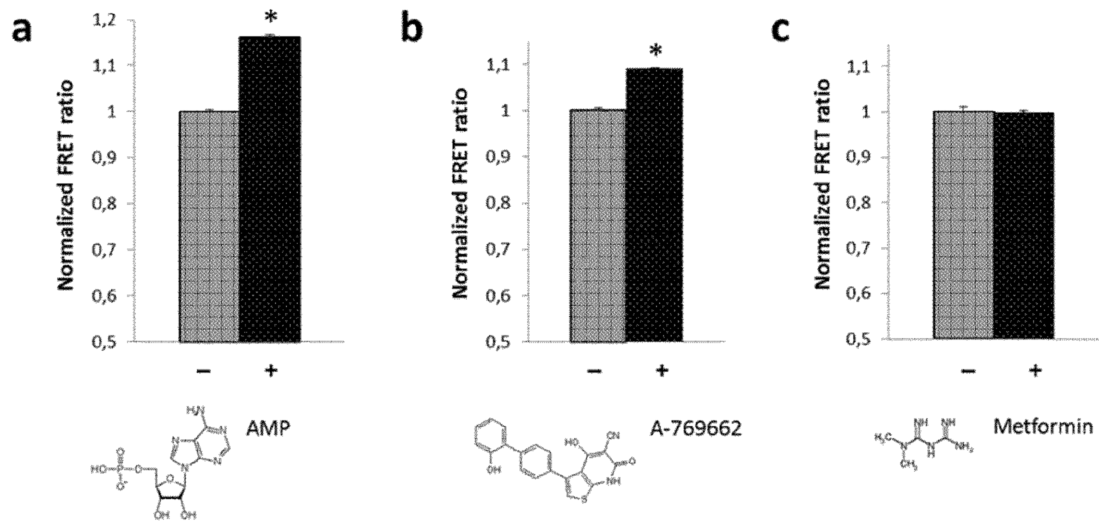


FIG. 7

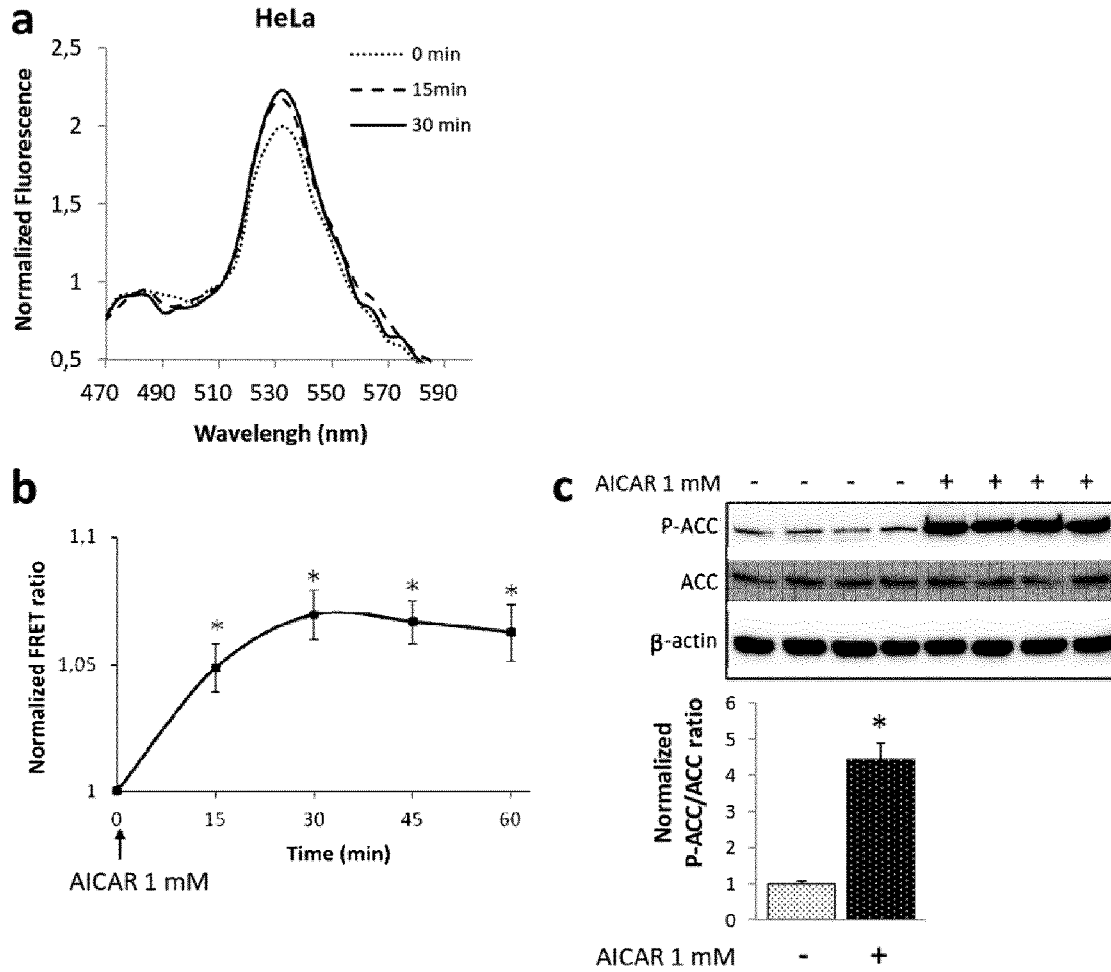


FIG. 8

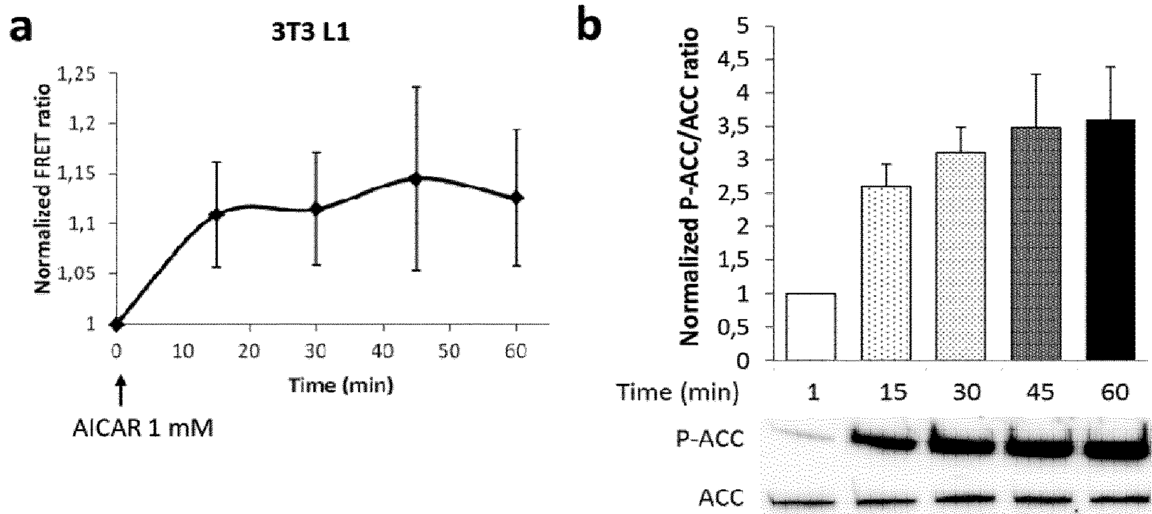


FIG. 9

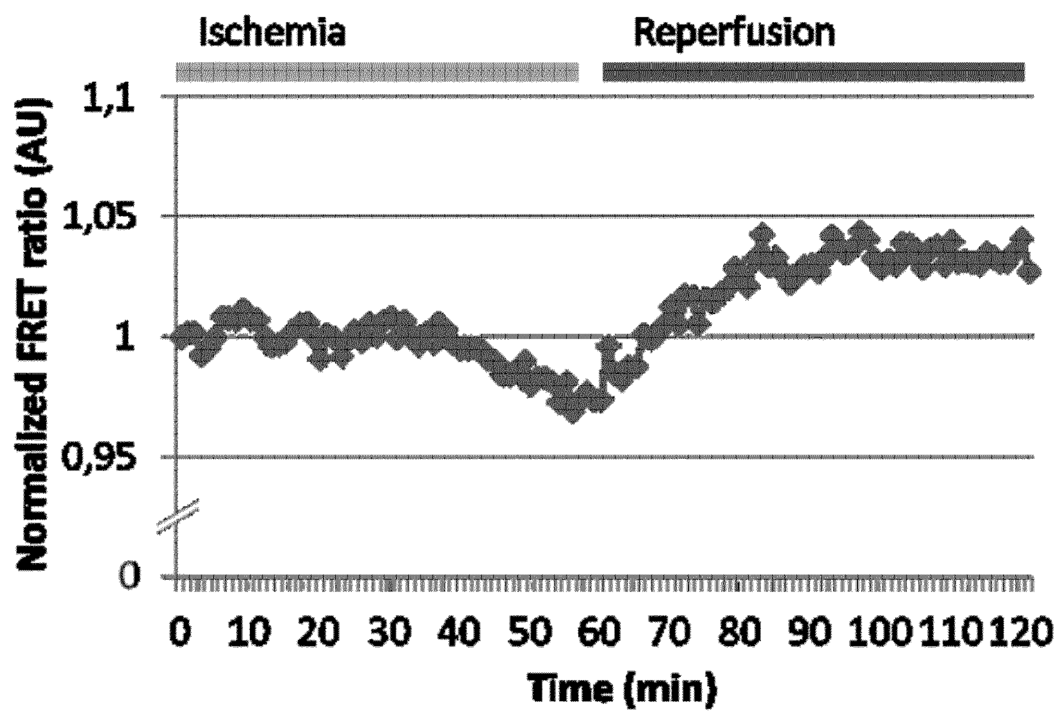


FIG. 10

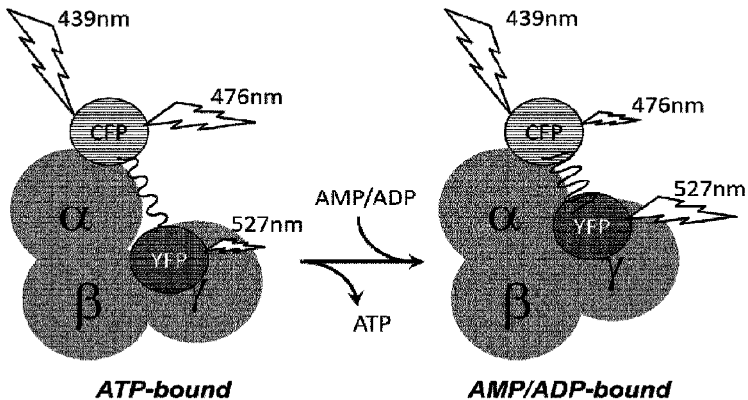
Original constructs
A B C D ... L

1st generation
- Selection
- AMPfret mutation
- FRET optimization (*FRET pair*)

2nd generation
- FRET optimization (*linker*)
- Selection
- FRET optimization (*FRET pair*)
- AMPfret mutation

1.0
1.0 mutants
1.1
A deletion
A α-helix deletion
C deletion
C α-helix
2.0
2.1
2.1 mutants

FRET pair:
eCFP/YFP
mseCFP$_{\Delta 11}$/cpVenus
eCFP/YFP
mseCFP$_{\Delta 11}$/cpVenus


439nm
476nm
CFP
527nm
YFP
α
β
γ
ATP-bound
AMP/ADP
ATP
439nm
476nm
CFP
527nm
YFP
α
β
γ
AMP/ADP-bound