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(71) **Demandeur/Applicant:**
CELLECTIS, FR

(72) **Inventeurs/Inventors:**
JUILLERAT, ALEXANDRE, US;
BERTONATI, CLAUDIA, FR;
VALTON, JULIEN, US;
DUCHATEAU, PHILIPPE, FR;
POIROT, LAURENT, FR

(74) **Agent:** BORDEN LADNER GERVAIS LLP

(54) **Titre : PROCEDE D'INGENIERIE DE LYMPHOCYTES T SENSIBLES A DE MULTIPLES SIGNAUX D'ENTREE A DES FINS D'IMMUNOTHERAPIE**
(54) **Title: METHOD OF ENGINEERING MULTI-INPUT SIGNAL SENSITIVE T CELL FOR IMMUNOTHERAPY**

(57) **Abrégé/Abstract:**
The present invention relates to a method to engineer immune cell for immunotherapy. In particular said immune cells are engineered with chimeric antigen receptors, which be activated by the combination of hypoxia and ligand extracellular binding as input signals. The invention also relates to new designed chimeric antigen receptors which are able to redirect immune cell specificity and reactivity toward a selected target exploiting the ligand-binding domain properties and the hypoxia condition. The present invention also relates to cells obtained by the present method, in particular T-cells, comprising said chimeric antigen receptors for use in cancer treatments.

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(71) Applicant: **CELLECTIS** [FR/FR]; 8 rue de la Croix Jarry, F-75013 Paris (FR).

(72) Inventors: **JUILLERAT, Alexandre**; 38, rue de l'Amiral Mouchez, F-75014 Paris (FR). **BERTONATI, Claudia**; 18, rue Gravilliers, F-75003 Paris (FR). **VALTON, Julien**; rue Victor Hugo, F-94220 Charenton le Pont (FR). **DUCHATEAU, Philippe**; Bateau Fawen, Quai des Dames, F-91210 Draveil (FR). **POIROT, Laurent**; 4, rue de Torcy, F-75018 Paris (FR).

(74) Agent: **ZACCO DENMARK A/S**; Arne Jacobsens Allé 15, DK-2300 Copenhagen S (DK).

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(54) **Title**: METHOD OF ENGINEERING MULTI-INPUT SIGNAL SENSITIVE T CELL FOR IMMUNOTHERAPY

(57) **Abstract**: The present invention relates to a method to engineer immune cell for immunotherapy. In particular said immune cells are engineered with chimeric antigen receptors, which be activated by the combination of hypoxia and ligand extracellular binding as input signals. The invention also relates to new designed chimeric antigen receptors which are able to redirect immune cell specificity and reactivity toward a selected target exploiting the ligand-binding domain properties and the hypoxia condition. The present invention also relates to cells obtained by the present method, in particular T-cells, comprising said chimeric antigen receptors for use in cancer treatments.

Method of engineering multi-input signal sensitive T cell for immunotherapy

Field of the description

5 The present invention relates to a method to engineer T cell for immunotherapy. In particular said T cells are engineered in order to be activated by the combination of input signals. The present invention relates to new designed chimeric antigen receptors which are able to redirect immune cell specificity and reactivity toward a selected target exploiting the ligand-binding domain properties. The present invention also relates to cells obtained by the present method, preferably
10 comprising said chimeric antigen receptors for use in therapeutic or prophylactic treatment.

Background of the invention

Adoptive immunotherapy, which involves the transfer of autologous antigen-specific T cells generated *ex vivo*, is a promising strategy to treat viral infections and cancer. The T cells used for
15 adoptive immunotherapy can be generated either by expansion of antigen-specific T cells or redirection of T cells through genetic engineering (Park, Rosenberg et al. 2011). Transfer of viral antigen specific T cells is a well-established procedure used for the treatment of transplant associated viral infections and rare viral-related malignancies. Similarly, isolation and transfer of tumor specific T cells has been shown to be successful in treating melanoma.

20 Novel specificities in T cells have been successfully generated through the genetic transfer of transgenic T cell receptors or chimeric antigen receptors (CARs) (Jena, Dotti et al. 2010). CARs are synthetic receptors consisting of a targeting moiety that is associated with one or more signaling domains in a single fusion molecule. In general, the binding moiety of a CAR consists of an antigen-binding domain of a single-chain antibody (scFv), comprising the light and heavy variable
25 fragments of a monoclonal antibody joined by a flexible linker. Binding moieties based on receptor or ligand domains have also been used successfully. The signaling domains for first generation CARs are derived from the cytoplasmic region of the CD3zeta or the Fc receptor gamma chains. First generation CARs have been shown to successfully redirect T cell cytotoxicity, however, they failed to provide prolonged expansion and anti-tumor activity *in vivo*. Signaling domains from co-
30 stimulatory molecules including CD28, OX-40 (CD134), ICOS and 4-1BB (CD137) have been added alone (second generation) or in combination (third generation) to enhance survival and increase

proliferation of CAR modified T cells. CARs have successfully allowed T cells to be redirected against antigens expressed at the surface of tumor cells from various malignancies including lymphomas and solid tumors (Jena, Dotti et al. 2010). However, for example, cancer cells are unstable and some cells may no longer possess the target antigen. These cells, referred to as antigen loss escape variants, escape destruction by the therapy and may continue to grow and spread unchecked. Cancer and healthy cells may express the same antigen although at different levels. In such case, having the possibility to combine at least two antigens in order for the engineered T cell to discriminate between healthy tissue and cancer cells would present extremely valuable advantage over actual technology for therapeutic purposes. Bispecific tandem CAR has already been described (International application: WO2013123061, US. Patent application: US20130280220). However, in this design the bispecific chimeric antigen receptor comprises (a) at least two antigen-specific targeting regions, (b) an extracellular spacer domain, (c) a transmembrane domain, (d) at least one co-stimulatory domain and (e) an intracellular signaling domain, wherein each antigen-specific targeting region comprises an antigen-specific single chain Fv (scFv) fragment, and binds a different antigen. Such design may theoretically still lead to the T-cell activation independently to the recognition and binding of both antigens as one cannot exclude that the binding of one single chain Fv may trigger activation. Kloss, Condomines et al. 2013 described another combinatorial antigen recognition approach. A CAR comprising a signaling domain mediated the recognition of one antigen and another receptor comprising a co-stimulatory domain specific for a second antigen are expressed at the surface of a T cell. This dual targeting approach facilitates augmented T cell reactivity against tumor positive for two antigens. However this approach alone fails to prevent T cell reactivity to single-positive tumors. To remedy this failure, search of adapted configuration of CAR are required.

To avoid the tuning of CAR used for the combinatorial antigen recognition, the inventors developed a system wherein activation of T cell is only induced through the combination of at least two signals. Each input signal alone does not induce the activation of T cell. Environmental signal integration by a modular AND gate within a CAR design may provide the ultimate strategy to insure safety and expand the number of surface antigens available for therapeutic purposes.

Logic gates are the basic building blocks in electronic circuits that perform logical operations. These have input and output signals in the form of 0's and 1's; '0' signifies the absence of signal while '1' signifies its presence. Similar to the electronic logic gates, cellular signals can serve as logic gates.

Synthetic biology applies many of the principles of engineering to the field of biology in order to create biological devices which can ultimately be integrated into increasingly complex systems. These principles include standardization of parts, modularity, abstraction, reliability, predictability, and uniformity (Andrianantoandro, Basu et al. 2006). The application of engineering principles to biology is complicated by the inability to predict the functions of even simple devices and modules within the cellular environment. Some of the confounding factors are gene expression noise, mutation, cell death, undefined and changing extracellular environments, and interactions with the cellular context (Andrianantoandro, Basu et al. 2006). Thus, while synthetic biology offers much promise in developing systems to address challenges faced in the fields of manufacturing, environment and sustainability, and health and medicine, the realization of this potential is currently limited by the diversity of available parts and effective design frameworks (Wang, Wei et al. 2013).

Summary of the invention

The present invention is drawn to apply synthetic biology principles such as logic “AND GATE” to immune cell technology in order for the cells to be stimulated and/or activated only by the combinations of at least two input signals (Figure 1). In particular, the present invention relates to a method of engineering immune cell for immunotherapy by render them sensitive to the combination of at least two input signals. Said input signals can be external stimuli such as hypoxia or the recognition of a ligand, preferably via the expression at the surface of the cell of a specific chimeric antigen receptor capable of recognizing said ligand. According to the present invention, the recognition of the input signals allow the combination of at least two transmitter domains which activate immune cell response, preferably via signaling protein. Each transmitter protein is independently inactive and thus does not activate immune cell response. Only the combination of these two transmitter domains allows T cell activation. The transmitter domains can be as non limiting examples, protease and an anchored membrane substrate domain comprising a protease cleavage site linked to a signaling protein, split proteins, scaffolding proteins, domains capable of dimerizing, autoinhibited protein with compound able to retrieve the inhibition, complementation of a prior inactivated gene. The present invention also relates to new design of chimeric antigen receptors, cells comprising said chimeric antigen receptors or obtained by the method of the invention, and therapeutic treatment using said engineered immune cell.

Brief description of the figures and tables

Figure 1: Logic "AND GATE" synthetic biology principle. Input (1,2) can be antigens expressed by tumor cells (and/or healthy cells) and /or tumor microenvironements. Output corresponds to the resulting activation of the immune cell.

5 **Figure 2: AND GATE: Tumor antigen-driven dimerization and activation of receptor tyrosine kinase (RTK)-based chimeric antigen receptors.** The simultaneous presence of two tumor cell ligands co localized at the tumor cell surface will drive the dimerization of two heterodimeric receptor tyrosine kinase-based chimeric antigen receptors, and lead to their activation via transphosphorylation. On both CARs, the transmitter domains are maintained in an inactive state 10 by autoinhibition (e.g.: the kinase active site is masked by autoinhibitory domain). The presence of two tumor cell ligands colocalized at the tumor cell surface enable driving dimerization of the two CARs resulting in the relief of kinase autoinhibition and enabling activation of transmitter domain via transphosphorylation or its interaction with other molecules as non limiting examples.

Figure 3: AND GATE: prior inactivated gene complementation: in a simplified example two 15 different tumor cell ligands can be recognized by two different CARs whose cytoplasmatic domains comprehend two different domains. Simultaneously the knock out of a key gene (GOI) in the signal pathway of the T cell has been performed. Upon the co-localization of the two CARs followed the recognition of the tumor ligand cells the first CAR can activate a factor which will enable the reactivaction of the GOI necessary to the transmission of the signal mediated by the 20 second CAR.

Figure 4: AND GATE: Protease system. The simultaneous presence of two tumor cell ligands will activate the two CARs. The intracellular domain of the first CAR comprehends a protease target sequence linked to a signaling protein; the intracellular domain of the second CAR is harboring a protease. Each CAR independently is not activated by the presence of the single tumor ligand cell, 25 the activation derives from the co-localization of the two CARs dues to the presence of both tumor ligand cells. The co-localization of the two CARs allows their activation mediated by the cleavage of the target sequence protease and the following release of the signaling protein.

Figure 5: AND GATE: Split protein system. The simultaneous presence of two tumor cell ligands will activate the two CARs. The intracellular domain of the first CAR comprehends a fragment of 30 the "signaling domain" and C or N domain of the intein. The cytoplasmatic domain of the second CAR is harboring the complementary intein domain plus the complementary signaling domain

fragment. Each CAR independently is not activated by the presence of the single tumor ligand cell, the activation derives from the co-localization of the two CARs dues to the presence of both tumor ligand cells. The co-localization of the two CARs allows their activation through the reconstitution of the full active split intein driving the reconstitution of the complete active form of the signaling protein which could initiate different pathways of activation of the T cell. Examples of signaling proteins are ZAP70, SH2 domains, and kinase domain.

Figure 6: AND GATE: Split protein system and release of the signaling protein. The simultaneous presence of two tumor cell ligands will activate the two CARs. The intracellular domain of the first CAR comprehends the C terminal inactive fragment of the "signaling protein" and C or N domain of the intein. The intracellular domain of the second CAR is harboring a dimerization domain which could homodimerize with an additional multi-domain. This multi-domain is constituted by the second intein domain and the N domain of the signaling protein fragment. Each CAR independently is not activated by the presence of the single tumor ligand cell, the activation derives from the co-localization of the two CARs dues to the presence of both tumor ligand cells. The co-localization of the two CARs allows their activation through the reconstitution of the full active split intein driving the reconstitution of the complete active form of the signaling protein, which could be released into the cytoplasm to initiate the activation of the T cell.

Figure 7: AND GATE: Kinase based split protein system. The simultaneous presence of two tumor cell ligands will activate the two CARs. The intracellular domain of the first CAR comprehends a signaling protein binding region and C or N domain of a split kinase. The intracellular domain of the second CAR is harboring the complementary kinase domain. Each CAR independently is not activated by the presence of the single tumor ligand cell, the activation derives from the co-localization of the two CARs dues to the presence of both tumor ligand cells. The co-localization of the two CARs allows their activation through the reconstitution of the full active kinase which could be phosphorylated hence start the activation of the T cell. Example of split kinase could be LCK.

Figure 8: AND GATE: Kinase based split protein system activation in *trans*. The simultaneous presence of two tumor cell ligands will activate the two CARs. The intracellular domain of the first CAR comprehends a signaling binding region and C or N domain of a split kinase. The intracellular domain of the second CAR is harboring the complementary kinase domain. Each CAR independently is not activated by the presence of the single tumor ligand cell; the activation derives from the co-localizatin of the two CARs dues to the presence of both tumor ligand cells.

The co-localization of the two CARs allows their activation through the reconstitution of the full active kinase which could cause a conformational modification on the signaling protein binding region allowing the binding of the signaling protein which could be activated by a phosphorylation in trans.

5 **Figure 9: AND GATE: Protease based split system and re-localization of the signaling protein.** The simultaneous presence of two tumor cell ligands will activate the two CARs. The intracellular domain of the first CAR comprehends the C or N domain of a split protease, a protease target sequence and the signaling protein. The intracellular domain of the second CAR is harboring the complementary split protease domain. Each CAR independently is not activated by the presence 10 of the single tumor ligand cell; the activation derives from the co-localization of the two CARs dues to the presence of both tumor ligand cells. The co-localization of the two CARs allows their activation through the reconstitution of the full active protease which could cleave the protease target sequence and cause the release of the signaling protein.

Figure 10: AND GATE: Protease based split system using three CARs. The simultaneous presence 15 of three tumor cell ligands will activate the CARs. The intracellular domain of the first CAR comprehends the protease target sequence and the signaling protein. The intracellular domains of the second and third CARs are constituted by the two complementary split protease domains. Each CAR independently is not activated by the presence of the single tumor ligand cell; the activation derives from the co-localization of three CARs dues to the presence of the three tumor 20 ligand cells. The co-localization of three CARs allows their activation through the reconstitution of the full active protease which could cleave the protease target sequence and cause the release of the signaling protein.

Figure 11: AND GATE: Scaffolding protease based system. The simultaneous presence of two tumor cell ligands will activate the two CARs. The intracellular domain of the first CAR 25 comprehends a first protein domain. The intracellular domain of the second CAR is harboring a second protein domain. Each CAR independently is not activated by the presence of the single tumor ligand cell, the activation derives from the co-localization of the two CARs dues to the presence of both tumor ligand cells. The co-localization of the two CARs allows their activation through the binding of the protein domain 1 and 2 to the inactive scaffolding protein. Upon the 30 complex binding the active form of the scaffolding protein is reconstituted and the T cell can be activated. Examples of scaffolding proteins are Carma1, SP76, hemITAM, DLG1, KSR.

Figure 12: AND GATE: Based on double activation of engineered heterodimeric domains. The simultaneous presence of two tumor cell ligands will activate the two CARs. The intracellular domain of the first CAR comprehends a first transmitter binding domain. The intracellular domain of the second CAR is harboring a second transmitter binding domain. Each Car independently is not activated by the presence of the single tumor ligand cell, the activation derives from the co-localization of the two CARs dues to the presence of both tumor ligand cells. The co-localization of the two CARs allows the activation of the two transmitter binding domains (e.g. phosphorylation and post-translation modifications) which can trigger the recruitment of a transmitter which can activate the T cell.

Figure 13: AND GATE: Autoinhibition system: induced activation upon competitive binding. The simultaneous presence of two tumor cell ligands will activate the transmitter. On the first CAR, the transmitter domain is maintained in an inactive state by autoinhibition (e.g.: by interaction with a “shielding” protein or antibody). The colocalization of the second CAR upon binding to its ligand will displace the shielding molecule to itself on a domain of higher affinity (intermolecular displacement). The “unshielded” transmitter can then be activated (e.g.: by post-translational modifications or interaction with other molecules).

Figure 14: AND GATE: Autoinhibition system: induced activation upon enzymatic cleavage of the inhibition domain. The simultaneous presence of two tumor cell ligands will activate the transmitter. On the first CAR, the transmitter domain is maintained in an inactive state by autoinhibition (e.g.: by interaction with a “shielding” protein or antibody). The colocalization of the second CAR upon binding to its ligand will bring a protease domain in close proximity of a protease target sequence present on the first car and thus allow to displace the shielding molecule. The “unshielded” transmitter can then be activated (e.g.: by post-translational modifications or interaction with other molecules).

Figure 15: AND GATE: Receptor binding and external stimuli to induce activation of the transmitter protein. The simultaneous binding of a CAR to its tumor cell ligand and the exposition of the engineered T cell to a tumor cell extracellular stimulus will activate the transmitter. External stimuli encompass variation in concentration of metabolites, small molecules, peptide, small proteins (chemokines, cytokines) and physico/chemical conditions (pH, hypoxia, redox potential).

Figure 16: AND GATE: Hypoxia dependent activation system in the presence of one tumor antigen.

The simultaneous presence of engineered T cells with one tumor cell ligand in an oxygen depleted environment, triggers T cells activation. To enable such logical AND gate activation system, T cell are engineered to harbor an oxygen-inducible synthetic activation pathway. Such synthetic pathway is made of three different elements including an engineered transcription factor sensitive to oxygen concentration (OxiTF), a synthetic promoter specific for the OxiTF driving the expression of the third element, a chimeric antigen receptor (CAR I). The OxiTF is design to activate a synthetic genetic element encoding a CAR specific for tumor antigen within engineered T cells. Upon solid tumor encounter, engineered T cells detect oxygen depletion and trigger CAR I production. Cell surface exposure of CARI enables the recognition of tumor antigen that eventually triggers T cells activation and proliferation via the activation and co-stimulatory domains present within CARI.

Figure 17: AND GATE: Hypoxia dependent activation system in the presence of two tumor antigen.

The simultaneous presence of engineered T cells with two tumor cell ligands in an oxygen depleted environment, triggers T cells activation. To enable such logical AND gate activation system, T cells are engineered to harbor an oxygen-inducible synthetic activation pathway. Such synthetic pathway is made of four different elements including an engineered transcription factor sensitive to oxygen concentration (OxiTF), a synthetic promoter specific for the OxiTF driving the expression of the third element, a chimeric antigen receptor (CAR II) specific for tumor antigen II. The system is completed with a fourth element, consisting in a constitutively expressed CARI, specific for tumor antigen I. The OxiTF is design to activate a synthetic genetic element encoding a CARII within engineered T cells. Upon solid tumor encounter, engineered T cells detect oxygen depletion and trigger CAR I production. Cell surface exposure of CARII along with CARI enables the recognition of tumor antigen II in addition to the pre-existing CARI-tumor antigen I complex. Simultaneous presence of both CAR/Tumor antigen complexes eventually triggers T cells activation and proliferation via the activation and co-stimulatory domains present within CARI and II.

Figure 18: AND GATE: Illustration of the AND GATE principle applied to the T cell system activation. The extracellular of a CAR contains two ligand-binding domains that that exist under two conformations (“active” and “inactive”). In absence of the two tumor cell ligands, the equilibrium is strongly displaced towards the “inactive form”. Only the simultaneous binding of

the two ligand-binding domains to their respective tumor cell ligands (two inputs) will trigger a positive signal to the intracellular domain of the CAR (output).

Figure 19: AND NOT GATE: General schema. Illustration of the AND NOT GATE principle applied to the T cell system activation. The simultaneous presence of two tumor cell ligands and the absence of an healthy cell ligands will trigger a positive output. The input 1 and 2 corresponds to the presence of a tumor cell ligand while the third input should not be a healthy cell ligand. The first and the second CAR have a co-stimulatory cytoplasmatic domains while the third CAR is harboring an inhibitory domain whose inhibitor effect will be blocked in case of non recognition of the healthy cell ligand.

Figure 20. **Generation of two types of LCKs to inhibit and to stimulate the T cell signaling cascade** The first CAR will recognize an antigen of an healthy cell with an inhibitory domain which will stimulate the transcription of a form constitutively negatively regulated of $LCK^{(-)}$. This first CAR will be coupled with a second one which contains a co-stimulatory domains which will activate the transcription of $LCK^{(+)}$ form, producing an high level of activation of the T cell.

Figure 21. Control of T cell activation through CAR-mediated regulation of CARMA1 protein. The stimulation of TCR after recognizing the antigen is linked to the recruitment of CD28 which leads to the activation of PKCθ, which in turn phosphorylates and activates CARMA1. CARMA1 constitutes a crucial signalosome for the transmission of the T cell Receptor (TCR) signaling and in general for T cell activation. CARMA1 recruits different proteins forming a multi –protein complexes that finally can activate two different signaling cascades: NF-κB and c-jun N-terminal kinase (JNK).

Figure 22. Functioning of the HIF hypoxia System. In normoxia (high O_2), HIFα is hydroxylated by HIFα-specific prolyl hydroxylases (PHD1-3) which are oxygen sensing. Hydroxylation triggers poly-ubiquitylation of HIFα and targets the latter for proteosomal degradation by an E3 ubiquitin ligase. In hypoxia (low O_2), occur an inhibition of hydroxylation via TCA cycle intermediates, a stabilization of the HIFα protein and an impairment of HIF transcriptional activity.

Figure 23. **Different chimeric antigen receptor (CAR) architectures carrying oxygen-sensitive domain such as HIFα**. At left, a single-chain CAR (scCAR) carries on the same and unique chain the extracellular binding domain (here scFv), the oxygen domain (ex: HIF1) and the activation and co-activation domains. At right is shown as an exemplary conformation a multi-chain CAR (mcCAR)

wherein the α-chain carries the scFv and the oxygen domain; the β-chain carries the co-stimulatory domain and the γ-chain carries the activation domain(s).

Figure 24. A) Surface presentation of chain-HIF1 (a.a. 380-630) versus α-chain WT α-in normoxia or hypoxia. The surface exposition of CAR T-cells having HIF1 in hypoxia is similar to the one of
5 control CAR T-cells (without α-HIF1), whereas the surface exposition is much reduced in normoxia condition, showing a good expression of CAR-αHIF1.

(B) Surface presentation of the α-chain-HIF1 (a.a. 380-630) versus α-chain WT after return from hypoxia to normoxia. The expression of CAR αHIF1 drops from hypoxia to normoxia condition. This is a reversible and dynamic system: in normoxia condition, the CAR expression is inhibited by
10 the degradation of α-HIF1 and α-chain polypeptides in a temporally manner, and in hypoxia condition (i.e. tumor environment), the α-HIF1 and α-chain polypeptides are expressed.

Figure 25. (A) Surface detection of α-HIF mcCAR versus control CAR in normoxia or hypoxia. In this experiment, less total RNA is used, the results obtained are similar to those of Figure 23;

(B) Induced cytotoxicity in normoxia. The control multi-chain CAR (without α-HIF1) shows a high
15 target cell killing, whereas the latter is null for the HIF-mcCAR in normoxia. In view of these results of cytotoxicity, as well as those of surface exposition, this indicates that the HIF system is fully functional within a chimeric antigen receptor.

Figure 26. Surface presentation of various α-chain-HIF versus WT α-chain in normoxia or hypoxia. (A) HIF1-mcCAR (a.a. 380-630) construct with the –EA – linker ; (B) HIF1-mcCAR (a.a. 344-
20 417) ; (C) HIF3-mcCAR (a.a. 480-571) ; (D-E-F): same as for (A-B-C) but return from hypoxia to normoxia.

All the results obtained here by lentiviral delivery demonstrate that both HIF1 and HIF3 systems are functional and behave similarly. Also, it is shown that different parts of the HIF protein can be used with or without linker.

25 Legends:

Sample	Conditions	Histogram
Isotype control	Hypoxia	solide line-Filled
α β γ	Normoxia	Dotted line
α β γ	Hypoxia	Dashed line
α-HIF β γ	Normoxia	Dotted line-Filled
α-HIF β γ	Hypoxia	Dashed line-Filled

Figure 27 (A) Schematic representation of the dual receptors logic AND gate. (B) Interaction of both membrane protein partners with their target ligand will trigger the colocalization of the intracellular interacting domains. (C) Release of the transmitter domain is triggering the output signal.

5 **Figure 28** **Schematic representation of the composition of both gate receptors.**

Figure 29 **Schematic representation of the molecular assembly strategy of the components for both gates, wherein the spacers are indicated**.

Figure 30 **Surface expression of 7 membrane protein partners**. GG83, GG111, GG121, GG152, GG153, GG155, GG156 and GG158 were tested ; intensity of the signal (++: very good, +: 10 good)..

Figure 31 **Expression of the lentiviral delivered RQR8 cassette by mRNA transfection of different transactivators.** These constructions are composed of a DNA binding domain (TetO or Gal4) and a transcription activation domain (VP64 or NF-kB),are transfected and are tested. The data obtained clearly indicated the expression of the lentiviral delivered RQR8 cassette by mRNA 15 transfection of the adequate transactivator.

Figure 32 **T7 endonuclease assay demonstrating targeted mutagenesis at the endogenous loci using the designed TALEN**: all the 3 panels A, B, C depict the knock-out (KO) of enzymes involved in T-cell signaling and/or functioning, such as LAT, LCK, ZAP70, LFA, TRAT or CD28. The data obtained clearly indicate a high level of targeted mutagenesis at all targeted loci using the 20 designed TALEN.

Figure 33 **Degranulation experiments following the Knock-outs of ZAP70**. The data obtained clearly indicate a strong staining decrease for the knock-out engineered T-cell relative to WT T-cells

Figure 34 **Schematic representation of bi-specific CAR (biCAR) functioning**. The bi-specific 25 CAR (biCAR) is composed of two CARs (biCAR 1 and biCAR2) whose scFv have specific affinity to two different target cell antigens. When only one of these scFc binds to its specific antigen, there is no activation of the CAR and therefore no killing of the cell. When both scFv bind to their specific antigens, an activation of the CAR occurs and the target cell killed.

Table 1: Proteins that interact with the CARMA1 Signalosome

Table 2: CARMA1 Phosphorylation Sites

Detailed description of the invention

5 The ability to control functional responses in adoptive T cell therapy is a key issue. In such therapeutic strategies, T cells are engineered by expressing surface-exposed chimeric antigen receptor (CAR) that achieves high tumor specific target cell recognition. However, to control and minimize potential toxic off-target effects, design of multi-input systems is highly desirable.

Depending of the type of "*input signal*", "*the combination of at least two transmitter domains*" 10 can be performed by direct or indirect means.

For instance in case of the split-ubiquitin system, the combination of two input signals, which are the recognition of two different cell target ligands by the scFv from the 2 CARs, makes possible the co-localization of the two transmitter domains, i.e. the C- and N- terminal parts of the ubiquitin enzyme, therefore the activity of the latter can occur and a signal is produced.

15 The hypoxia HIFalpha system functions in a more indirect manner especially in regard to "*the combination of transmitters*". The combination of two input signals occurs between one hypoxia external signal and the other from the cell target ligand recognition by scFv of the CAR. At this stage, occurs a cascade of reactions such as inhibition of phosphorylation of HIF prolyl-hydroxylase, stabilization of the HIF-1α subunit, upregulatation of several genes to promote 20 survival in low-oxygen conditions, and ultimately binding of HIF-1 to HIF-responsive elements (HREs).

By "*activation of immune cell*", it is meant that the combination of two inputs signals triggers (directly or indirectly) the combination of two transmitters domains, which in its turn generates a 25 signal (positive or negative) to the immune cell carrying the CAR, preferably by transduction means.
Consequently, a signal is emitted for the CAR expression and finally the lysis of the tumoral cells may happen.

30

Methods of engineering immune cells

The inventors developed methods of engineering such immune cells based on the rational combination of regulatory modules in artificial circuits for performing tasks, including complex binary computation operations based on logic gates. The term “gate” is used to refer to a device or molecular mechanism that produces a particular (predetermined) output in response to two or more input signals. According to the present invention, the logical AND gate refers to the immune cell activation, in particular T cell cytotoxicity against a target cell through the combination of different transmitter domains and activation of specific proteins (signaling proteins) resulting from the combination of at least two input signals. As non limiting examples, each signal can act together or separately to activate protein function or to remove an inhibiting protein. In another particular embodiment, said input signal can be the output signal resulting from prior input signals. In particular, the present invention relates to a method of engineering an immune cell for immunotherapy, in particular, method of engineering an immune cell for targeting specifically a cell comprising:

(a) Providing an immune cell;

(b) Engineering said immune cell to render said cell sensitive to at least two input signals such that the combination of input signals induces combination of at least two transmitter domains which results in activation of said immune cell, wherein each transmitter domain alone does not activate said immune cell.

This method is different from the combinatorial antigen recognition system described in (Kloss, Condomines et al. 2013) wherein said transmitter domains are signaling domain and co-stimulatory domains of a chimeric antigen receptor as described below, said signaling domain alone can activate T cell activation.

Input signal: Recognition of a ligand by immune cell

In a particular embodiment, said input signal can be the recognition of a ligand by said engineered immune cell, in particular by chimeric antigen receptor expressed at the surface of said engineered immune cell.

The chimeric antigen receptor (CAR) according to the present invention comprises an extracellular ligand-binding domain and an intracellular domain, more particularly, an extracellular ligand binding domain, a transmembrane domain and an intracellular domain.

The term "extracellular ligand-binding domain" as used herein is defined as an oligo- or
5 polypeptide that is capable of binding a ligand. Preferably, the domain will be capable of interacting with a cell surface molecule. For example, the extracellular ligand-binding domain may be chosen to recognize a ligand that acts as a cell surface marker on target cells associated with a particular disease state. Thus examples of cell surface markers that may act as ligands include those associated with viral, bacterial and parasitic infections, autoimmune disease and cancer
10 cells. In particular, the extracellular ligand-binding domain can comprise an antigen binding domain derived from an antibody against an antigen of the target. As non limiting examples, the antigen of the target can be a tumor-associated surface antigen as described above.

The extracellular ligand-binding domain can also comprise a peptide binding an antigen of the target, a peptide or a protein binding an antibody that binds an antigen of the target, a peptide or
15 a protein ligand such as a growth factor, a cytokine or a hormone as non limiting examples binding a receptor on the target, or a domain derived from a receptor such as a growth factor receptor, a cytokine receptor or a hormone receptor as non limiting examples, binding a peptide or a protein ligand on the target. Preferably the target is a cell or a virus.

In a preferred embodiment, said extracellular ligand-binding domain is a single chain antibody
20 fragment (scFv) comprising the light (V_L) and the heavy (V_H) variable fragment of a target antigen specific monoclonal antibody joined by a flexible linker. Other binding domain than scFv can also be used for predefined targeting of lymphocytes, such as camelid single-domain antibody fragments, receptor ligands like a vascular endothelial growth factor polypeptide, an integrin-binding peptide, heregulin or an IL-13 mutein, antibody binding domains, antibody hypervariable
25 loops or CDRs as non limiting examples.

In another preferred embodiment, said extracellular binding domain can be a DARPin (designed ankyrin repeat protein). DARPins are genetically engineered antibody mimetic proteins typically exhibiting highly specific and high-affinity target protein binding. They are derived from natural ankyrin proteins and comprise at least three, usually four or five repeat motifs of these proteins.
30 DARPins are small, single domain proteins which can be selected to bind any given target protein with high affinity and specificity (Epa, Dolezal et al. 2013; Friedrich, Hanauer et al. 2013; Jost, Schilling et al. 2013). According to the present invention, DARPins can be engineered to comprise

multiple antigen recognition sites. Thus, said DARPins can be used to recognize a series of consecutive different antigens as well as a unique antigen. Thus, the present invention relates to a method comprising providing an immune cell, and expressing at the surface of said immune cell chimeric antigen receptor which comprises a designed ankyrin repeat protein capable of
5 recognizing at least one specific ligand, preferably at two specific ligands.

As non limiting example, the ligand of the target can be a tumor-associated surface antigen, such as ErbB2 (HER2/neu), carcinoembryonic antigen (CEA), epithelial cell adhesion molecule (EpCAM), epidermal growth factor receptor (EGFR), EGFR variant III (EGFRvIII), CD19, CD20, CD30, CD40, disialoganglioside GD2, GD3, C-type lectin-like molecule-1 (CLL-1), ductal-epithelial mucine, gp36,
10 TAG-72, glycosphingolipids, glioma-associated antigen, β-human chorionic gonadotropin, alphafetoprotein (AFP), lectin-reactive AFP, thyroglobulin, RAGE-1, MN-CA IX, human telomerase reverse transcriptase, RU1, RU2 (AS), intestinal carboxyl esterase, mut hsp70-2, M-CSF, prostase, prostase specific antigen (PSA), PAP, NY-ESO-1, LAGA-1a, p53, prostein, PSMA, surviving and telomerase, prostate-carcinoma tumor antigen-1 (PCTA-1), MAGE, ELF2M, neutrophil elastase,
15 ephrin B2, CD22, insulin growth factor (IGF1)-I, IGF-II, IGFI receptor, mesothelin, a major histocompatibility complex (MHC) molecule presenting a tumor-specific peptide epitope, 5T4, ROR1, Nkp30, NKG2D, tumor stromal antigens, the extra domain A (EDA) and extra domain B (EDB) of fibronectin and the A1 domain of tenascin-C (TnC A1) and fibroblast associated protein (fap), LRP6, melamona-associated Chondroitin Sulfate Proteoglycan (MCSP), CD38/CS1, MART1,
20 WT1, MUC1, LMP2, Idiotype, NY-ESO-1, Ras mutant, gp100, proteinase 3, bcr-abl, tyrosinase, hTERT, EphA2, ML-TAP, ERG, NA17, PAX3, ALK, Androgen receptor ; a lineage-specific or tissue specific antigen such as CD3, CD4, CD8, CD24, CD25, CD33, CD34, CD79, CD116, CD117, CD135, CD123, CD133, CD138, CTLA-4, B7-1 (CD80), B7-2 (CD86), endoglin, a major histocompatibility complex (MHC) molecule, BCMA (CD269, TNFRSF 17), or a virus-specific surface antigen such as
25 an HIV-specific antigen (such as HIV gp120); an EBV-specific antigen, a CMV-specific antigen, a HPV-specific antigen, a Lasse Virus-specific antigen, an Influenza Virus-specific antigen as well as any derivate or variant of these surface markers. In specific cases, the ligand that the chimeric antigen receptor recognizes is present on the surface of a target cell, particularly cancer cell or viral cell. In some embodiments, the ligand that the chimeric antigen receptor recognizes is
30 present in a tumor microenvironment. In some aspects of the invention, the ligand that the chimeric antigen receptor recognizes is a growth factor.

In a preferred embodiment, said input signals can be the recognition of at least two different ligands by chimeric antigen receptors expressed at the surface of engineered immune cells. Thus,

the immune cell of the present method can be engineered by expressing at the surface of said immune cell at least two Chimeric Antigen Receptors (CAR) each comprising an extracellular domain capable of recognizing different ligands and an intracellular domain comprising transmitter domain. The combination of at least the two input signals corresponding to the recognition of different ligands by each extracellular domains of said CARs allows the combination of at least two transmitter domains and thus activation of said immune cell.

In a particular embodiment, the present method comprises the expression of at least two CARs comprising an extracellular ligand binding domain capable of recognizing the combination of several ligands such as non limiting examples the combination of HER2, MUC1, CD44, CD49f and/or epCAM to target breast cancer, the combination of mesothelin, folate receptor-alpha, CD44 and/or CD133 to target ovarian cancer cells, the combination of HER2 and IL13R-alpha2 for the treatment of glioblastoma, CD19 and CD20, Cd19 and CD22, CD20 and LI-CAM, LI-CAM and GD2, EGFR and LICAM, EGFR and C-MAT, EGFR and HER2, C-MET and HER2, EGFR and ROR1. In specific cases, at least one of the ligand that the chimeric antigen receptor recognizes is present on the surface of a target cell, particularly cancer cell. In some embodiments, at least one of the ligand that the chimeric antigen receptor recognizes is present in a tumor microenvironment. In some aspects of the invention, at least one of the ligand that the chimeric antigen receptor recognizes is a growth factor. In some embodiments, the first ligand is specific for an antigen present on a cancer cell surface and the second ligand is present in a tumor microenvironment.

The CAR according to the present invention is expressed on the surface membrane of the cell. Thus, the CAR comprises a transmembrane domain. The distinguishing features of appropriate transmembrane domains comprise the ability to be expressed at the surface of a cell, preferably in the present invention an immune cell, in particular lymphocyte cells or Natural killer (NK) cells, and to interact together for directing cellular response of immune cell against a predefined target cell. The transmembrane domain can be derived either from a natural or from a synthetic source. The transmembrane domain can be derived from any membrane-bound or transmembrane protein. As non limiting examples, the transmembrane polypeptide can be a subunit of the T cell receptor such as α, β, γ or δ, polypeptide constituting CD3 complex, IL2 receptor p55 (α chain), p75 (β chain) or γ chain, subunit chain of Fc receptors, in particular Fcγ receptor III or CD proteins. Alternatively the transmembrane domain can be synthetic and can comprise predominantly hydrophobic residues such as leucine and valine. In a preferred embodiment said transmembrane domain is derived from the human CD8 alpha chain (e.g. NP_001139345.1). Said transmembrane domain can also be a CD8 transmembrane domain (alpha and beta chains). Said Transmembrane

domain can be engineered to create obligated hetero or homodimers. In particular embodiment said CARs can comprise transmembrane domains or intracellular domains which can only dimerize after ligand recognition. Another example of transmembrane domain can be NKG2-D receptor. NKG2D (natural killer cell group 2D) is a C-type lectin-like receptor expressed on NK cells, $\gamma\delta$-TcR$^+$
5 T cells, and CD8$^+\alpha\beta$-TcR$^+$ T cells (Bauer, Groh et al. 1999). NKG2D is associated with the transmembrane adapter protein DAP10 (Wu, Song et al. 1999), whose cytoplasmic domain binds to the p 85 subunit of the PI-3 kinase. In a preferred embodiment, two complementary architectures of CAR recogizing two different ligands can be expressed at the surface of the immune cell, a first CAR comprising an ITAM motif as described below and a second CAR
10 comprising NKG2-D triggering an alternative signaling pathway.

Another example of transmembrane domain can be a receptor tyrosine kinase. Receptor tyrosine kinase are cell surface receptors involved in different critical cellular regulatory process including cell proliferation, cell differentiation, cell survival, cell migration, as well as cell cycle control. Receptor tyrosine kinase comprises an extracellular domain, a single transmembrane helix and an
15 intracellular domain comprising tyrosine kinase function that is most of time autoregulated by additional carboxy-terminal and juxtamembrane domains. Activation of receptor tyrosine kinase is generally elicited by ligand-mediated dimerization. Thanks to their bivalence, growth hormone ligand has the capacity to simultaneously interact with two receptor monomers and promotes dimerization. Such dimerization induces the activation of intracellular kinase domains through
20 conformational changes followed by trans-phosphorylation of different tyrosines located within their intracellular domain. The different phosphotyrosines generated eventually serve as docking site for the recruitment of downstream signaling partners that activate the cellular regulatory pathways. In a preferred embodiment, said CAR can comprise the extracellular domain, transmembrane, and/or the intracellular domain of a receptor tyrosine kinase, preferably selected
25 from the group consisting of TrkA, c-Kit, FGFR and EGFR/Erb. Said tyrosine kinase transmembrane domain and/or intracellular domain can be linked to an extracellular ligand binding domain and intracellular domain according to the present invention (Figure 2). In particular embodiment, said engineered cells comprise different CARs comprising different transmembrane domains.

Said transmembrane domain can also be an integrin. Integrins are heterodimeric integral
30 membrane proteins composed of a α and β chains which combined together form the LFA-1 (integrin lymphocyte function-associated antigen-1) which is expressed on all leukocytes. LFA-1 plays a central role in leukocyte intercellular adhesion through interactions with its ligand, ICAMs

1-3 (intercellular adhesion molecules 1 through 3), and also it has an important role in lymphocyte co-stimulatory signaling (Chen and Flies 2013). The molecular details of the binding of LAF-1 to its immunoglobulin ICAM-1 are quite known allowing a careful engineering of LAF-1 binding site. The affinity of α_L domain for ICAM-1 is regulated by the displacement of its C-terminal helix which is conformational linked to alterations of specific loops in LAF-1. The active and low conformations differ of 500 and 10,000 folds. It is also interesting to note that two types of antagonists are known for LFA-1 and their mechanism of action is known. Integrin cell surface adhesion receptors can transmit a signal from the outside to inside but also *viceversa*. There are cytoskeletal proteins as Talin which binds to the integrin tail LFA-1 to transfer a message from inside to outside.

Integrins are part of the immunological synapse and their spatial/location in the synapse seems to be strategically to the creation of an effective response to the T cell stimulation caused by the recognition of exposed antigens on the antigen presenting cells (Singleton, Roybal et al. 2009).

Indeed here we expose the idea to use the integrin scaffold to modulate the response of T cell exposing CAR. The integrin can be used to boost the activity of CAR engineered T cell enhancing its natural role of adhesion between the T cell and the tumoral cell allowing for a higher concentration of perforin and granzyme at the immunological synapse. More we can imagine to use the integrin to create a new generation of CAR whose scaffold could be a fusion between the integrin scaffold (i.e. the alpha and the beta chains but also other chains) and scFV domains (or any other type of antigen receptors). The possibility to modulate the 3D conformation of the integrins respects with the presence of small molecules in the cytoplasm creates remarkably opportunities. Indeed the integrin is naturally present in two forms: one low affinity form which hinders the active domains (the one responsible for the binding of the natural ligand i.e. ICAM) at the membrane surface and one active form with very high affinity for the natural ligand which is exposing the active domains in the extracellular milieu.

The transmembrane domain can further comprise a stalk region between said extracellular ligand-binding domain and said transmembrane domain. The term “stalk region” (also named hinge region) used herein generally means any oligo- or polypeptide that functions to link the transmembrane domain to the extracellular ligand-binding domain. In particular, stalk region are used to provide more flexibility and accessibility for the extracellular ligand-binding domain. A stalk region may comprise up to 300 amino acids, preferably 10 to 100 amino acids and most preferably 25 to 50 amino acids. Stalk region may be derived from all or part of naturally occurring molecules, such as from all or part of the extracellular region of CD8, CD4, CD28 or RTK, or from all

or part of an antibody constant region. Alternatively the stalk region may be a synthetic sequence that corresponds to a naturally occurring stalk sequence, or may be an entirely synthetic stalk sequence.

The intracellular domain of the CAR according to the present invention comprises a transmitter
5 domain. Indeed, according to the present invention, the input signals induce the combination of the transmitter domains leading to immune cell activation. In a particular embodiment, said transmitter domain is a signaling protein, and the combination of signaling protein function induces immune cell activation. In another particular embodiment, said transmitter domains are at least two molecules which can interact together, and the interaction induce immune cell
10 activation.

In a particular embodiment, said CAR can be a multi-chain CAR comprising at least a transmembrane polypeptide which comprises at least one extracellular ligand binding domain; and a transmembrane polypeptide comprising at least one transmitter domain such that said polypeptides assemble together to form a multi-chain Chimeric Antigen Receptor
15 (PCT/US2013/058005). Said multi-chain CAR can comprise several extracellular ligand binding domains, to simultaneously bind different ligands. In particular, said different extracellular ligand-binding domains can be placed on different transmembrane polypeptides composing the multi-chain CAR. In another embodiment, the present invention relates to a population of multi-chain CARs comprising each one different extracellular ligand binding domains.

20 In a particular embodiment, said chimeric antigen receptor comprises at least:
- an extracellular ligand binding domain capable of recognizing said specific ligand;

- a transmembane domain;

- an intracellular domain comprising at least an activation and co-activation domains and an oxygen-sensitive domain.

25 According to another embodiment, said extracellular domain contains additionally a hinge.

According to another embodiment, the scFv contained in said extracellular binding domain are directed to the CD19, 5T4, ROR1, CD123 or CD33 cell target antigens, and have respectively at least and identity of over 80%, preferably 90% or more preferably 95% with SEQ ID NO: 32, 35, 38 ; SEQ ID NO: 33 ; SEQ ID NO:34 ; SEQ ID NO:36 and SEQ ID NO:37.

According to another embodiment, said hinge is chosen from CD8a, IgG1 or EpoR-D2, and have an identity of over 80%, preferably 90% or more preferably 95% with respectively SEQ ID NO:39, 40 and 41.

According to another embodiment, said transmembrane domain is chosen from CD8a, 4-1BB, 5 DAP10, CD28 or FceRIalpha, and have an identity of over 80%, preferably 90% or more preferably 95% with respectively SEQ ID NO:42, 43, 44, 45 and 46.

According to another embodiment, said oxygen-sensitive domain is chosen between HIF1 alpha or HIF3 alpha, and have an identity of over 80%, preferably 90% or more preferably 95% with respectively SEQ ID NO:22, 23, 85, and SEQ ID NO:26, 27.

10 According to another embodiment, said intracellular domain contains a linker chosen amongst CD3zeta, FceRIg, CD28, 4-1BB, OX40, DAP10, CD28, CD275, HVEM, LIGHT, CD40L, GITR, TIM1, SLAM, CD2, TLT-2, LAG3, DAP12, CD84, CD244, CD229, LTBR and CD278, and have an identity of over 80%, preferably 90% or more preferably 95% with respectively SEQ ID NO:47 to 70.

According to another embodiment, said said activation domain is CD3zeta, and said activation 15 domain is chosen between 4-1BB or CD28.

Complementation of a prior inactivated gene

The transmitter proteins can also complement a prior inactivated gene or activate a gene in the nucleus to complement a prior inactivated gene. Thus, following combination of input signals, the combination of two transmitter signals allows the complementation of the inactivated gene and 20 thus the activation of the T cell.

Domains involved in the formation of the immune synapse can be used as target for gene inactivation, and thus for complementation of this gene. Said immune cell inactivated for this gene can be used to engineer cell according to the present invention. Thus, following combination of input signals, the combination of transmitter domains induces the expression of a gene capable of 25 complementing said inactivating gene. As non limiting examples, said domains which participate to the formation of the immunological synapse or to the transfer of the signal include as non-limiting examples: LCK, ZAP70, Itk, LAT, SLP76, GADS, GRB2, PLC-γ1, or VAV1. Other examples can be DOK1 and DOK2 proteins which negatively control the T cell receptor signaling by recruiting other negative regulators as RAS GTP, SHIP1 and CSK. Transcription factors modulated by the 30 immunological synapse domains can also be activate to complement inactivated cell. Said transcription factors include as non-limiting examples: NFAT (nuclear factor of activated T cells),

NF-κb (nuclear factor kappa-light chain enhancer of activated B cells), mTOR, AP1/2, ERK1/2, C-MAF. For example, ZAP-70 (Zeta-chain-associated protein kinase 70) is a protein normally expressed near the surface membrane of T cells. It is part of the T cell receptor, and plays a critical role in T cell signaling. Following antigen recognition by CAR comprising the CD3 zeta signaling domain in immune cell, ZAP70 binds to the CD3 zeta domain inducing activation of immune cell response. Thus, in inactivated ZAP70 gene T cell, the antigen recognition of only one antigen by CAR comprising CD3 zeta domain does not induce T cell activation. However, the recognition of a second ligand by another CAR comprising an intracellular ZAP70 domain can complement the prior inactivated ZAP70 gene and thus allows the activation of the T cell via CD3 zeta (Figure 3).

Protease system

Transmitter domains according to the present invention can be a protease and a substrate protein comprising a signaling protein linked to a membrane anchoring domain via a protease cleavage site. The combination of the two transmitter domains results in activation of the immune cell. Indeed, cleavage of the substrate protein by the protease results in the release of signaling protein and thus in immune cell activation (Figure 4). Said membrane anchoring domain can be a terminal extension which anchors the substrate protein to the membrane of the cell. In particular embodiment, said substrate protein is a part of the intracellular domain of a chimeric antigen receptor. Said protease can be as non-limiting examples: TEV protease, Factor Xa, thrombin, engineered viral poteases, enterokinase and HRV3C.

Split-protein based system

In another embodiment, the transmitter domains are split proteins. This system is based on protein complementation assays wherein a functional molecule is dissected into two non-functional fragments. Functionality is restored when the fragments are reassembled by attached protein-protein interaction domains. The functional molecule used in the protein complementation assays can be an active enzyme, or a signaling protein. Said split proteins encompass, as non-limiting examples, split kinases, split proteases and split inteins.

In a particular embodiment, said split proteins are split inteins which can reassemble together and restore the functionality of the intein. Inteins are internal protein sequences that catalyze a protein-splicing reaction, which precisely excise the intein sequence and join the flanking sequence with a peptide bond. Split intein is any intein in which the N-terminal domain of the intein and the C-terminal domain of the intein are not directly linked via a peptide bond. Natural

split inteins have been identified in cyanobacteria and archaea, but split inteins can also be created artificially by separating an intein sequence into two fragments (International application WO2013/045632). According to the present invention, the protein splicing reaction precisely excises the intein sequence and joins the flanking sequence to reconstitute a signaling protein which induces immune cell activation (Figure 5). In a particular embodiment, said signaling protein can be released upon spilt intein reassembly (Figure 6).

In another particular embodiment, said split proteins can be split kinases which can assemble together to reconstitute a functional kinase (Figure 7 and 8). Said kinase can phosphorylate a signaling protein to induce immune cell activation. In a particular embodiment, said kinases can be as non limiting examples: CaMKII, Lck, PKCq, HPK1, PKθ, IKKβ, CK1α which will phosphorylate the serine residues on the linker region of the CARMA1 protein inducing NF-κβ and JNK signaling pathway.

In another particular embodiment, said split proteins can be split-protease which can interact together to form a functional protease as described above (Figure 9 and 10). Said protease can interact with a substrate protein and cleave the target protease site to release the signaling protein.

Scaffolding systems

Scaffold proteins are crucial regulators of many key signaling pathways. By Scaffold protein, it is meant a protein able to interact and/or bind with multiple members of a signaling pathway, tethering them into complexes. In the present invention, the transmitter domains can be members of the signaling pathway which can recruit a scaffold protein. This assembly may be able to enhance signaling specificity and efficiency by increasing the proximity and effective concentration of components in the scaffold complex resulting in the activation of the immune cell. As non limiting example, a scaffold protein can bind a protein kinase and its substrate thereby ensuring specific kinase phosphorylation or said scaffold protein can result in allosteric changes of the signaling members. Said scaffold protein can regulate signal transduction, can help localize pathway components (organized in complexes) to specific areas of the cell such as the plasma membrane, can coordinate signaling feedbacks, or can protect activated molecules from inactivation. Said scaffold protein according to the present invention can be as non-limiting example SH2 domain as in SYK tyrosine kinase or ZAP70 which can recognize and bind different ITAM domains (transmitter domains) as described for C-type lectin and hemITAM (Figure 11) or CARMA-1 as described in example 2.

Double activation system

Transmitter domains can also be a homo or heterodimeric proteins, in particular an intracellular domain of a receptor which can dimerize with another transmitter domain such as another intracellular domain of a receptor or a cytosolic protein. The dimerization of these transmitter
5 domains transduces a signal downstream. One example of signaling proteins involving homo- or hetero-dimerization of proteins can be a tyrosine kinase receptor as described above involving the JAK/Stat signaling pathway. Activation of such components is generally elicited by ligand-mediated dimerization. Said transmitter domains that homodimerize can be engineered to form obligated heterodimer. In a particular embodiment, said CARs can comprise the transmembrane, and
10 optionally the intracellular domains of a receptor tyrosine kinase, preferably selected from the group consisting of TrkA, c-Kit, FGFR and EGFR/Erb. The recognition of the ligands induces the dimerization of the receptor and thus activation of the signaling protein resulting in immune cell activation (Figure 12).

Autoinhibited system

15 Transmitter domains can also be a non-activate form of an autoinhibited molecule. Autoinhibited compounds may exist in an autoinhibited state or an active state. The autoinhibited state results in perturbed catalytic function of the protein, or perturbs the ability of the protein to interact with another ligand. An autoinhibited state typically occurs in the absence of phosphorylation of the kinase. Activation of such autoinhibited protein can involve a conformational change of the
20 compound. This conformational change can be the consequence of the interaction with another compound. Said inhibitory compounds can be allosteric inhibitory compounds. Allosteric inhibitory compounds bind and form a specific association with an autoinhibited compound so as to preserve a conserve non activated conformational state of the autoinhibited compound. Autoinhibition can be relieved by interacting with another transmitter domain which can have a
25 higher affinity binding (Figure 13) or which can induce for examples covalent modifications (e.g. de-/phosphorylation) of the interaction region or proteolysis (Figure 14). As non limiting examples, said inhibitors can be class I and II of p21 activated kinase (pak) inhibitor, Rho activated protein inhibitors, autoinhibites non receptor serine/threonine kinase inhibitors, phosphatase inhibitor and autoinhibited small GTPase effector inhibitors.

30

The combination of transmitter domains according to the present invention is responsible for intracellular signaling following the binding of extracellular ligand binding domain and results in the activation of the immune cell and immune response. In other words, the signaling protein is responsible for the activation of at least one of the normal functions of the engineered immune cell. For example, the function of a T cell can be a cytolytic activity or helper activity including the secretion of cytokines. Thus, the term "signaling protein" refers to a protein which transduces the transmitter domain function signal and directs the cell to perform a specialized function. In a particular embodiment, said transmitter domain can be a signaling protein. Transmission of the signals can result from: protein/protein interactions, protein/DNA interaction, protein/RNA interaction, protein/small molecule interaction, post translational protein modification, conformational change, subcellular relocalization.

In particular, the signaling protein can complement a prior inactivated gene or activate a gene in the nucleus to complement a prior inactivated gene. Domains involved in the formation of the immune synapse can be used as target for gene inactivation, and thus for complementation of this gene. Said immune cell inactivated for this gene can be used to engineer cell according to the present invention. Thus, following combination of input signals, the combination of transmitter domains induces the expression of a gene capable of complementing said inactivating gene.

In another particular embodiment, the signaling protein can activate a gene in the nucleus. Examples of signaling protein can be members of NFAT transcription factor family which are inducible factor that could bind the intereukin-2 promoter in activated T cells. The regulation of NFAT proteins involves metabolites and proteins such as calcium, calcineurin and Homer scaffolding proteins. Said signaling protein can be an activated engineered form of NFAT avoiding regulation by calcineurin and Homer proteins. Said signaling protein can be a NF-κB engineered to avoid sequestration in the cytoplasm by Iκb allowing activation of T cells. Said signaling protein can also be the expression of the three IKK subunits (IKKα, IKKβ, IKKy). Reconstituted IKK complex activated NF-κB pathway, by triggering the ubiquitination of the IκB. Also the activation of the JNK signaling could be triggered through the direct expression of signaling protein AP-1 (transcription factor). In another particular embodiment, said signaling protein can be an engineered transcription activator like effector (TALE) binding domain that will specifically target and activate transcription of the same gene as for the NFAT and NF-kb.

In another particular embodiment, said signaling protein can inhibit a signaling pathway through protein-protein interaction or can activate a gene in the nucleus to inhibit a signaling pathway.

Said signaling protein can be vaccinia H1 related proteins (VHR) a member of the mitogen-activated protein kinase phosphatases (MKPs) family which dephosphorylates and inactivates an extracellular signal regulated kinases (ERK) signaling proteins.

According to the present invention, said transmitter domains or signaling protein induced by 5 transmitter domains can be a signal transducing domain. Preferred examples of signal transducing domain for use in a CAR can be the cytoplasmic sequences of the T cell receptor and co-receptors that act in concert to initiate signal transduction following antigen receptor engagement, as well as any derivate or variant of these sequences and any synthetic sequence that has the same functional capability. Signal transduction domain comprises two distinct classes of cytoplasmic 10 signaling sequence, those that initiate antigen-dependent primary activation, and those that act in an antigen-independent manner to provide a secondary or co-stimulatory signal. Primary cytoplasmic signaling sequence can comprise signaling motifs which are known as immunoreceptor tyrosine-based activation motifs of ITAMs. ITAMs are well defined signaling motifs found in the intracytoplasmic tail of a variety of receptors that serve as binding sites for 15 syk/zap70 class tyrosine kinases or lck. Examples of ITAM used in the invention can include as non limiting examples those derived from TCRzeta, FcRgamma, FcRbeta, FcRepsilon, CD3gamma, CD3delta, CD3epsilon, CD5, CD22, CD79a, CD79b and CD66d.

In particular embodiment the signal transduction domain of the CAR of the present invention comprises a co-stimulatory signal molecule. A co-stimulatory molecule is a cell surface molecule 20 other than an antigen receptor or their ligands that is required for an efficient immune response. “Co-stimulatory ligand” refers to a molecule on an antigen presenting cell that specifically binds a cognate co-stimulatory molecule on a T cell, thereby providing a signal which, in addition to the primary signal provided by, for instance, binding of a TCR/CD3 complex with an MHC molecule loaded with peptide, mediates a T cell response, including, but not limited to, proliferation 25 activation, differentiation and the like. A co-stimulatory ligand can include but is not limited to CD7, B7-1 (CD80), B7-2 (CD86), PD-L1, PD-L2, 4-1BBL, OX40L, inducible costimulatory igand (ICOS-L), intercellular adhesion molecule (ICAM, CD30L, CD40, CD70, CD83, HLA-G, MICA, M1CB, HVEM, lymphotoxin beta receptor, 3/TR6, ILT3, ILT4, an agonist or antibody that binds Toll ligand receptor and a ligand that specifically binds with B7-H3. A “co-stimulatory molecule” refers to the cognate 30 binding partner on a T cell that specifically binds with a co-stimulatory ligand, thereby mediating a co-stimulatory response by the cell, such as, but not limited to proliferation. Co-stimulatory molecules include, but are not limited to an MHC class I molecule, BTLA and Toll ligand receptor. Examples of costimulatory molecules include CD27, CD28, CD8, 4-1BB (CD137), OX40, CD30, CD40,

PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3 and a ligand that specifically binds with CD83 and the like.

External stimuli

In another aspect of the invention, the input signal can be an external stimulus (Figure 15). Said
5 external stimuli encompass as non limiting examples variation upon presence of a tumor cell in the microenvironment of the engineered T cell of small molecules, peptide, small proteins, (chemokines, cytokines) and physicochemical conditions such as pH, hypoxia, redox potential. Redox regulatory elements can be oxygen or nitrogen such as reactive oxygen or nitrogen specied (NS and RNS) as non limiting examples.

10 In a particular embodiment, said external stimulus can be hypoxic environment.

In a preferred embodiment, the response to said hypoxia condition is triggered by the alpha hypoxia inducible factor 1 (HIF-1α) or by the alpha hypoxia inducible factor 3 (HIF-3α).

In a more preferred embodiment, the said HIF-1α polypeptide sequence has over 80% identity, preferably 90% identity or more preferably 95% identity with SEQ ID NO. 5 or to SEQ ID NO.22-23, or the said HIF-3α polypeptide sequence has over 80% identity, preferably 90% identity or more preferably 95% identity with SEQ ID NO. 26-27.

Indeed, local tissue hypoxia is associated with many different disease states including certain tumors, certain inflammatory processes and conditions of neovascularization. Solid tumours, in particular, show relatively aberrant vascularization that causes intermittent/absent perfusion leading to hypoxia. A multifaceted adaptive response to hypoxia is facilitated by the stabilization
15 and accumulation of the alpha subunit of hypoxia inducible factor 1 (HIF-1). Under normoxia, HIF1α is inhibited through the hydroxylation of specific proline residues located in its C-terminal region. Such hydroxylation is known to promote recruitment of VHL, an E3 ubiquitin ligase that triggers ubiquitinylation of HIF1α and its degradation by proteasome. In hypoxic conditions, HIF-1α forms a complex with its binding partner, aryl hydrocarbon receptor nuclear translocator
20 (ARNT), as well as the p300/CBP transcriptional coactivators that bind to the hypoxia response element (HRE) in the untranslated regions of hypoxically regulated genes. This complex induces the transcription of genes that serve to maintain cellular homeostasis in the face of hypoxic conditions. For example, the HIF-1/p300/CBP complex plays a role in inducing expression of genes such as those encoding erythropoietin, which leads to erythropoiesis; vascular endothelial cell
25 growth factor (VEGF), which is a primary mediator of angiogenesis; iNOS and heme oxygenase,

which play roles in vasodilation; and the glucose transporter and glycolytic enzymes, which play roles in anaerobic metabolism. Beside HIF-1, the HIF-3 α system (ref Uniprot: Q9Y2N7 for human sequence) is also contemplated in the present invention. Also involved in adaptive response to hypoxia, its action is known to suppress hypoxia-inducible expression of HIF1A and EPAS, and to
5 binds to core DNA sequence 5'-TACGTG-3' within the hypoxia response element (HRE) of target gene promoters. Its expression and characterization in human kidney are presented in Hara et al. (2001).

In a particular aspect of the invention, said input signal can be the hypoxic microenvironment which is detected by the engineered cell. Thus, according to the method of the present invention,
10 the immune cell can be engineered to be sensitive to hypoxic environment. Said immune cell is engineered to trigger cytotoxicity via activation of synthetic hypoxia dependent activation pathways. In particular, said immune cell can be engineered to induce expression of a transmitter domain under hypoxia inducible promoter. Said hypoxia inducible promoter can be composed of HRE. The consensus sequence of HRE is (G/C/T)ACGTGC(G/C). Usually, multiple copies of HREs
15 appear in a hypoxia inducible promoter. In a preferred embodiment, said hypoxia inducible promoter is composed of HREs and a basal promoter such as SV40 promoter. For example, said multiple copies of HRE can be derived from the PGK-1 promoter, the EPO, GAPDH, VEGF and survivin promoters.

In another particular embodiment, said immune cell is engineered by expressing a transcription
20 factor sensitive to oxygen (oxiTF) and incorporating within the cell a synthetic promoter specific for the OxiTF driving the expression of a transmitter domain (Figure 16). Said OxiTF can be an engineered transcription factor such as TAL effector, Zinc finger effector, CRISP effector, as non limiting examples, fused to an HIF1 alpha C-terminal domain. Under hypoxia, engineered immune cells sense oxygen depletion, in particular in tumor environment, and trigger the expression of a
25 transmitter domain which induces immune cell activation. Said transmitter domain can be a transmitter domain or more particularly a chimeric antigen receptor comprising transmitter domains as described above. Said chimeric antigen receptor comprises an extracellular ligand binding domain capable of recognizing a specific ligand and the recognition of said ligand by said CAR is the second input signal . Said method of engineering immune cells can further comprise the
30 step of expressing at the surface of said immune cell another chimeric antigen receptor. In a more preferred embodiment, such engineered cell can comprised a first CAR constitutively expressed at the surface of the immune cell and a second CAR expressed only in hypoxic conditions, the first

and second CARs comprising extracellular binding domains capable of recognizing two different ligands (Figure 17).

In another particular embodiment, the present invention encompasses a method of engineering an immune cell wherein the chimeric antigen receptor is a multi-chain CAR. In a preferred embodiment , α, β and γ chains ofsaid multi-chain CAR have respectively an identity of over 80%, preferably 90% identity or more preferably 95% identity with SEQ ID NO. 7, 3 and 4.

In another particular embodiment, the present invention relates to a method of engineering an immune cell comprising a step of expressing a CAR which comprises at least two extracellular
5 ligand binding domains. Bispecific tandem CAR previously described (International application: WO2013123061, US. Patent application: US20130280220), may theoretically still lead to the T cell activation independently to the recognition and binding of both antigen as one cannot exclude that the binding of one single chain Fv may trigger activation. So to avoid these drawbacks, the inventors sought to design new bispecific CAR comprising at least two extracellular ligand binding
10 domains, which cannot induce T cell activation by the binding of only one ligand. Said CAR can comprise another domain which perturbs the ability the signaling function of the CAR, or perturbs the ability of the protein to interact with another ligand. The recognition of at least two ligands by the extracellular ligand binding domains can involve a conformational change of the CAR and thus the transduction of the signal of the CAR. The conformational change can occur for example by
15 the interaction with a ligand which can have a higher affinity binding, such idiotypic antibody (Figure 18).

Other AND logic Gate system

In another aspect of the invention, the inventors also developed methods of engineering such
20 immune cells based on logic gates, wherein only the presence of one specific input signal and not the other produces a particular output in response (Figure 19). According to the present invention, the immune cell activation, in particular T cell cytotoxicity against a target cell is induced following the recognition of one of several input signals, in particular the recognition of a ligand on a cancer cell and not the recognition of a ligand present on the healthy cells. In particular, the present
25 invention relates to a method of engineering an immune cell for immunotherapy, in particular, method of engineering an immune cell for targeting specifically a cell comprising:

(a) Providing an immune cell;

(b) Engineering said immune cell to render said cell sensitive to at least one among several input signals such that only the specific input signal induces activation of said immune cell response.

5 In a particular embodiment, the present invention relates to a method of engineering immune cell by expressing at the surface of the cell at least one first CAR comprising an extracellular ligand binding domain capable of recognizing a ligand at the surface of a tumor cell and another CAR comprising an extracellular ligand binding domain capable of recognizing a ligand at the surface of healthy cell, such that the recognition of the ligand at the surface of a healthy cell inhibits the 10 activation of immune cell via an inhibitory transmitter domain, while the recognition of the ligand at the surface of the target cell induces the activation of the immune cell via transmitter domain. Inhibitory or activation transmitter domain can be derived from the SRC family kinase (SFK) member LCK. In a more preferred embodiment, inhibitory transmitter domain is a constitutively negatively regulated LCK, preferably comprising a mutation at the position Y394 (NCBI Reference 15 Sequence: NP_005347.3) and activation domain is a constitutively active LCK form, preferably which comprises a mutation at position Y505 (NCBI Reference Sequence: NP_005347.3) (see example 2 and Figure 20).

Delivery methods

The different methods described above involve expressing CAR at the surface of a cell. As non-20 limiting example, said CAR can be expressed by introducing CAR into a cell. CAR can be introduced as transgene encoded by one plasmidic vector. Said plasmid vector can also contain a selection marker which provides for identification and/or selection of cells which received said vector.

Polypeptides may be synthesized *in situ* in the cell as a result of the introduction of polynucleotides encoding said polypeptides into the cell. Alternatively, said polypeptides could be 25 produced outside the cell and then introduced thereto. Methods for introducing a polynucleotide construct into cells are known in the art and including as non limiting examples stable transformation methods wherein the polynucleotide construct is integrated into the genome of the cell, transient transformation methods wherein the polynucleotide construct is not integrated into the genome of the cell and virus mediated methods. Said polynucleotides may be introduced 30 into a cell by for example, recombinant viral vectors (e.g. retroviruses, adenoviruses), liposome and the like. For example, transient transformation methods include for example microinjection,

electroporation or particle bombardment. Said polynucleotides may be included in vectors, more particularly plasmids or virus, in view of being expressed in cells.

Chimeric Antigen Receptors, polynucleotides and vectors

5 The present invention also relates to a chimeric antigen receptor which comprises an extracellular ligand binding domain and an intracellular domain comprising a transmitter domain as described above. In particular, said transmitter domain is selecting from the group consisting of: protease, split protein, members of signaling pathway recruiting a scaffold protein, one monomer of the dimeric domain, an autoinhibited compound.

10 According to one embodiment, the chimeric antigen receptor comprises:
- the alpha-chain comprises extracellularly the CD8 hinge, FcRα as transmembrane domain, and intracellularly a part of FcRα combined with HIF1alpha or HIF3 alpha subunit;

- the beta-chain comprises the FcRβ as extracellular and transmembrane domain, and ΔITAM-41BB as intracellular co-stimulation domain;

15 - the gamma-chain comprises the FcRγ as transmembrane domain, and ΔITAM-CD3ζ as intracellular activation domain.

The present invention also relates to polynucleotides, vectors encoding the above described CAR according to the invention. The polynucleotide may consist in an expression cassette or expression vector (e.g. a plasmid for introduction into a bacterial host cell, or a viral vector such as
20 a baculovirus vector for transfection of an insect host cell, or a plasmid or viral vector such as a lentivirus for transfection of a mammalian host cell).

In a particular embodiment, the different nucleic acid sequences can be included in one polynucleotide or vector which comprises a nucleic acid sequence encoding ribosomal skip sequence such as a sequence encoding a 2A peptide. 2A peptides, which were identified in the
25 Aphthovirus subgroup of picornaviruses, causes a ribosomal "skip" from one codon to the next without the formation of a peptide bond between the two amino acids encoded by the codons (see (Doronina, Wu et al. 2008). By "codon" is meant three nucleotides on an mRNA (or on the sense strand of a DNA molecule) that are translated by a ribosome into one amino acid residue. Thus, two polypeptides can be synthesized from a single, contiguous open reading frame within an
30 mRNA when the polypeptides are separated by a 2A oligopeptide sequence that is in frame. Such

ribosomal skip mechanisms are well known in the art and are known to be used by several vectors for the expression of several proteins encoded by a single messenger RNA.

To direct, transmembrane polypeptide into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in 5 polynucleotide sequence or vector sequence. The secretory signal sequence is operably linked to the transmembrane nucleic acid sequence, i.e., the two sequences are joined in the correct reading frame and positioned to direct the newly synthesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the nucleic acid sequence encoding the polypeptide of interest, although certain secretory signal sequences 10 may be positioned elsewhere in the nucleic acid sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830).

Those skilled in the art will recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules. Preferably, the nucleic acid sequences of the present invention are codon-optimized for expression in 15 mammalian cells, preferably for expression in human cells. Codon-optimization refers to the exchange in a sequence of interest of codons that are generally rare in highly expressed genes of a given species by codons that are generally frequent in highly expressed genes of such species, such codons encoding the amino acids as the codons that are being exchanged.

20 Engineered immune cells

The present invention also relates to isolated cells or cell lines susceptible to be obtained by said method to engineer cells. In particular said isolated cell comprises at least one CAR as described above. In a preferred embodiment, said isolated cell comprises a population of CARs each one comprising different extracellular ligand binding domains. In particular, said isolated cell comprises 25 exogenous polynucleotide sequence encoding CAR.

In the scope of the present invention is also encompassed an isolated immune cell, preferably a T cell obtained according to any one of the methods previously described. Said immune cell refers to a cell of hematopoietic origin functionally involved in the initiation and/or execution of innate and/or adaptative immune response. Said immune cell according to the present invention can be 30 derived from a stem cell. The stem cells can be adult stem cells, non-human embryonic stem cells, more particularly non-human stem cells, cord blood stem cells, progenitor cells, bone marrow

stem cells, induced pluripotent stem cells, totipotent stem cells or hematopoietic stem cells. Representative human cells are CD34+ cells. Said isolated cell can also be a dendritic cell, killer dendritic cell, a mast cell, a NK-cell, a B-cell or a T cell selected from the group consisting of inflammatory T-lymphocytes, cytotoxic T-lymphocytes, regulatory T-lymphocytes or helper T-lymphocytes. In another embodiment, said cell can be derived from the group consisting of CD4+ T-lymphocytes and CD8+ T-lymphocytes. Prior to expansion and genetic modification of the cells of the invention, a source of cells can be obtained from a subject through a variety of non-limiting methods. Cells can be obtained from a number of non-limiting sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. In certain embodiments of the present invention, any number of T cell lines available and known to those skilled in the art, may be used. In another embodiment, said cell can be derived from a healthy donor, from a patient diagnosed with cancer or from a patient diagnosed with an infection. In another embodiment, said cell is part of a mixed population of cells which present different phenotypic characteristics. In the scope of the present invention is also encompassed a cell line obtained from a transformed T-cell according to the method previously described.

In another embodiment, said isolated cell according to the present invention comprises a polynucleotide encoding CAR.

Therapeutic applications

In another embodiment, isolated cell obtained by the different methods or cell line derived from said isolated cell as previously described can be used as a medicament. In another embodiment, said medicament can be used for treating cancer, autoimmune disease or infections in a patient in need thereof. In another embodiment, said isolated cell according to the invention or cell line derived from said isolated cell can be used in the manufacture of a medicament for treatment of a cancer, viral infection or autoimmune disease in a patient in need thereof.

In another aspect, the present invention relies on methods for treating patients in need thereof, said method comprising at least one of the following steps:

(a) providing an immune-cell obtainable by any one of the methods previously described;

(b) Administrating said transformed immune cells to said patient,

On one embodiment, said T cells of the invention can undergo robust *in vivo* T cell expansion and can persist for an extended amount of time.

Said treatment can be ameliorating, curative or prophylactic. It may be either part of an autologous immunotherapy or part of an allogenic immunotherapy treatment. By autologous, it is
5 meant that cells, cell line or population of cells used for treating patients are originating from said patient or from a Human Leucocyte Antigen (HLA) compatible donor. By allogeneic is meant that the cells or population of cells used for treating patients are not originating from said patient but from a donor.

Cells that can be used with the disclosed methods are described in the previous section. Said
10 treatment can be used to treat patients diagnosed with cancer, viral infection, autoimmune disorders or Graft versus Host Disease (GvHD). Cancers that may be treated include tumors that are not vascularized, or not yet substantially vascularized, as well as vascularized tumors. The cancers may comprise non solid tumors (such as hematological tumors, for example, leukemias and lymphomas) or may comprise solid tumors. Types of cancers to be treated with the multi-
15 chain CARs of the invention include, but are not limited to, carcinoma, blastoma, and sarcoma, and certain leukemia or lymphoid malignancies, benign and malignant tumors, and malignancies e.g., sarcomas, carcinomas, and melanomas. Adult tumors/cancers and pediatric tumors/cancers are also included.

It can be a treatment in combination with one or more therapies against cancer selected from the
20 group of antibodies therapy, chemotherapy, cytokines therapy, dendritic cell therapy, gene therapy, hormone therapy, laser light therapy and radiation therapy.

The administration of the cells or population of cells according to the present invention may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The compositions described herein may be
25 administered to a patient subcutaneously, intradermaliy, intratumorally, intranodally, intramedullary, intramuscularly, by intravenous or intralymphatic injection, or intraperitoneally. In one embodiment, the cell compositions of the present invention are preferably administered by intravenous injection.

The administration of the cells or population of cells can consist of the administration of 10^4-10^9
30 cells per kg body weight, preferably 10^5 to 10^6 cells/kg body weight including all integer values of cell numbers within those ranges. The cells or population of cells can be administrated in one or more doses. In another embodiment, said effective amount of cells are administrated as a single

dose. In another embodiment, said effective amount of cells are administrated as more than one dose over a period time. Timing of administration is within the judgment of managing physician and depends on the clinical condition of the patient. The cells or population of cells may be obtained from any source, such as a blood bank or a donor. While individual needs vary, 5 determination of optimal ranges of effective amounts of a given cell type for a particular disease or conditions within the skill of the art. An effective amount means an amount which provides a therapeutic or prophylactic benefit. The dosage administrated will be dependent upon the age, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment and the nature of the effect desired.

10 In another embodiment, said effective amount of cells or composition comprising those cells are administrated parenterally. Said administration can be an intravenous administration. Said administration can be directly done by injection within a tumor.

In certain embodiments of the present invention, cells are administered to a patient in conjunction with (e.g., before, simultaneously or following) any number of relevant treatment modalities, 15 including but not limited to treatment with agents such as antiviral therapy, cidofovir and interleukin-2, Cytarabine (also known as ARA-C) or nataliziimab treatment for MS patients or efaliztimab treatment for psoriasis patients or other treatments for PML patients. In further embodiments, the T cells of the invention may be used in combination with chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, 20 mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAMPATH, anti-CD3 antibodies or other antibody therapies, cytoxin, fludaribine, cyclosporin, FK506, rapamycin, mycoplienolic acid, steroids, FR901228, cytokines, and irradiation. These drugs inhibit either the calcium dependent phosphatase calcineurin (cyclosporine and FK506) or inhibit the p70S6 kinase that is important for growth factor induced signaling (rapamycin) (Henderson, 25 Naya et al. 1991; Liu, Albers et al. 1992; Bierer, Hollander et al. 1993). In a further embodiment, the cell compositions of the present invention are administered to a patient in conjunction with (e.g., before, simultaneously or following) bone marrow transplantation, T cell ablative therapy using either chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH, In another embodiment, the cell 30 compositions of the present invention are administered following B-cell ablative therapy such as agents that react with CD20, e.g., Rituxan. For example, in one embodiment, subjects may undergo standard treatment with high dose chemotherapy followed by peripheral blood stem cell transplantation. In certain embodiments, following the transplant, subjects receive an infusion

of the expanded immune cells of the present invention. In an additional embodiment, expanded cells are administered before or following surgery.

Other definitions

5 - Amino acid residues in a polypeptide sequence are designated herein according to the one-letter code, in which, for example, Q means Gln or Glutamine residue, R means Arg or Arginine residue and D means Asp or Aspartic acid residue.

- Nucleotides are designated as follows: one-letter code is used for designating the base of a nucleoside: a is adenine, t is thymine, c is cytosine, and g is guanine. For the degenerated 10 nucleotides, r represents g or a (purine nucleotides), k represents g or t, s represents g or c, w represents a or t, m represents a or c, y represents t or c (pyrimidine nucleotides), d represents g, a or t, v represents g, a or c, b represents g, t or c, h represents a, t or c, and n represents g, a, t or c.

- "As used herein, "nucleic acid" or "polynucleotides" refers to nucleotides and/or 15 polynucleotides, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), oligonucleotides, fragments generated by the polymerase chain reaction (PCR), and fragments generated by any of ligation, scission, endonuclease action, and exonuclease action. Nucleic acid molecules can be composed of monomers that are naturally-occurring nucleotides (such as DNA and RNA), or analogs of naturally-occurring nucleotides (e.g., enantiomeric forms of naturally-occurring 20 nucleotides), or a combination of both. Modified nucleotides can have alterations in sugar moieties and/or in pyrimidine or purine base moieties. Sugar modifications include, for example, replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, and azido groups, or sugars can be functionalized as ethers or esters. Moreover, the entire sugar moiety can be replaced with sterically and electronically similar structures, such as aza-sugars and carbocyclic 25 sugar analogs. Examples of modifications in a base moiety include alkylated purines and pyrimidines, acylated purines or pyrimidines, or other well-known heterocyclic substitutes. Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages. Nucleic acids can be either single stranded or double stranded.

- By chimeric antigen receptor (CAR) is intended molecules that combine a binding domain against 30 a component present on the target cell, for example an antibody-based specificity for a desired antigen (e.g., tumor antigen) with a T cell receptor-activating intracellular domain to generate a chimeric protein that exhibits a specific anti-target cellular immune activity. Generally, CAR

consists of an extracellular single chain antibody (scFv) fused to the intracellular signaling domain of the T cell antigen receptor complex zeta chain (scFv:ζ) and have the ability, when expressed in T cells, to redirect antigen recognition based on the monoclonal antibody's specificity.

- By “ delivery vector” or “ delivery vectors” is intended any delivery vector which can be used in the present invention to put into cell contact (i.e “contacting”) or deliver inside cells or subcellular compartments (i.e “introducing”) agents/chemicals and molecules (proteins or nucleic acids) needed in the present invention. It includes, but is not limited to liposomal delivery vectors, viral delivery vectors, drug delivery vectors, chemical carriers, polymeric carriers, lipoplexes, polyplexes, dendrimers, microbubbles (ultrasound contrast agents), nanoparticles, emulsions or other appropriate transfer vectors. These delivery vectors allow delivery of molecules, chemicals, macromolecules (genes, proteins), or other vectors such as plasmids, peptides developed by Diatos. In these cases, delivery vectors are molecule carriers. By “delivery vector” or “delivery vectors” is also intended delivery methods to perform transfection.

- The terms "vector" or “vectors” refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. A “vector” in the present invention includes, but is not limited to, a viral vector, a plasmid, a RNA vector or a linear or circular DNA or RNA molecule which may consists of a chromosomal, non chromosomal, semi-synthetic or synthetic nucleic acids. Preferred vectors are those capable of autonomous replication (episomal vector) and/or expression of nucleic acids to which they are linked (expression vectors). Large numbers of suitable vectors are known to those of skill in the art and commercially available.

Viral vectors include retrovirus, adenovirus, parvovirus (e. g. adenoassociated viruses), coronavirus, negative strand RNA viruses such as orthomyxovirus (e. g., influenza virus), rhabdovirus (e. g., rabies and vesicular stomatitis virus), paramyxovirus (e. g. measles and Sendai), positive strand RNA viruses such as picornavirus and alphavirus, and double-stranded DNA viruses including adenovirus, herpesvirus (e. g., Herpes Simplex virus types 1 and 2, Epstein-Barr virus, cytomegalovirus), and poxvirus (e. g., vaccinia, fowlpox and canarypox). Other viruses include Norwalk virus, togavirus, flavivirus, reoviruses, papovavirus, hepadnavirus, and hepatitis virus, for example. Examples of retroviruses include: avian leukosis-sarcoma, mammalian C-type, B-type viruses, D type viruses, HTLV-BLV group, lentivirus, spumavirus (Coffin, J. M., Retroviridae: The viruses and their replication, In Fundamental Virology, Third Edition, B. N. Fields, et al., Eds., Lippincott-Raven Publishers, Philadelphia, 1996).

- By “lentiviral vector” is meant HIV-Based lentiviral vectors that are very promising for gene delivery because of their relatively large packaging capacity, reduced immunogenicity and their

ability to stably transduce with high efficiency a large range of different cell types. Lentiviral vectors are usually generated following transient transfection of three (packaging, envelope and transfer) or more plasmids into producer cells. Like HIV, lentiviral vectors enter the target cell through the interaction of viral surface glycoproteins with receptors on the cell surface. On entry, 5 the viral RNA undergoes reverse transcription, which is mediated by the viral reverse transcriptase complex. The product of reverse transcription is a double-stranded linear viral DNA, which is the substrate for viral integration in the DNA of infected cells. By "integrative lentiviral vectors (or LV)", is meant such vectors as nonlimiting example, that are able to integrate the genome of a target cell. At the opposite by "non-integrative lentiviral vectors (or NILV)" is meant efficient gene 10 delivery vectors that do not integrate the genome of a target cell through the action of the virus integrase.

- Delivery vectors and vectors can be associated or combined with any cellular permeabilization techniques such as sonoporation or electroporation or derivatives of these techniques.

- by "mutation" is intended the substitution, deletion, insertion of up to one, two, three, four, five, 15 six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, twenty, twenty five, thirty, fourty, fifty, or more nucleotides/amino acids in a polynucleotide (cDNA, gene) or a polypeptide sequence. The mutation can affect the coding sequence of a gene or its regulatory sequence. It may also affect the structure of the genomic sequence or the structure/stability of the encoded mRNA.

20 - by "functional variant" is intended a catalytically active mutant of a protein or a protein domain; such mutant may have the same activity compared to its parent protein or protein domain or additional properties, or higher or lower activity.

-"identity" refers to sequence identity between two nucleic acid molecules or polypeptides. Identity can be determined by comparing a position in each sequence which may be aligned for 25 purposes of comparison. When a position in the compared sequence is occupied by the same base, then the molecules are identical at that position. A degree of similarity or identity between nucleic acid or amino acid sequences is a function of the number of identical or matching nucleotides at positions shared by the nucleic acid sequences. Various alignment algorithms and/or programs may be used to calculate the identity between two sequences, including FASTA, 30 or BLAST which are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and can be used with, e.g., default setting. For example, polypeptides having at least 70%, 85%, 90%, 95%, 98% or 99% identity to specific polypeptides described herein

and preferably exhibiting substantially the same functions, as well as polynucleotide encoding such polypeptides, are contemplated.

- The term "subject" or "patient" as used herein includes all members of the animal kingdom including non-human primates and humans.

5 - The term "Hypoxia" refers to a condition wherein the oxygen concentration is below normal levels for a particular tissue (such as a tumor). Hypoxia in a particular tissue as, compared to surrounding tissue, is referred to as relative tissue hypoxia. An example of relative tissue hypoxia is tumor hypoxia in which a tumor has lower levels of $p0_2$ than that of surrounding non-tumor tissue. In some examples of the disclosed methods, the level of oxygen is for example 10% or less
10 (for example, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1%), or for example 50 mmHg or less (for example, 45 mmHg, 40 mmHg, 35 mmHg, 30 mmHg, 25 mmHg, 20 mmHg, 15 mmHg, 10 mmHg, 5 mmHg, 4 mmHg, 3 mmHg, 2 mmHg, or 1 mmHg). The body as a whole (generalized hypoxia) or a region of the body (tissue hypoxia) may be deprived of adequate oxygen. Those of skill in the art would be familiar with the measurement of oxygen levels in biological systems and that oxygen
15 measurements may be expressed in "mmHg," wherein, for example, 10% 0_2 is equal to 76 mmHg and 1% 0_2 is equal to 7.6 mmHg.

- By "Transcription Activator like Effector (TALE)" it is meant a binding domain protein wherein sequence specificity is driven by a series of 33-35 amino acids repeats originating from *Xanthomonas* or *Ralstonia* bacterial proteins. These repeats differ essentially by two amino acids
20 positions that specify an interaction with a base pair (Boch, Scholze et al. 2009; Moscou and Bogdanove 2009). Each base pair in the DNA target is contacted by a single repeat, with the specificity resulting from the two variant amino acids of the repeat (the so-called repeat variable dipeptide, RVD). TALE binding domains may further comprise an N-terminal translocation domain responsible for the requirement of a first thymine base (T_0) of the targeted sequence and a C-
25 terminal domain that containing a nuclear localization signals (NLS). A TALE nucleic acid binding domain generally corresponds to an engineered core TALE scaffold comprising a plurality of TALE repeat sequences, each repeat comprising a RVD specific to each nucleotides base of a TALE recognition site. In the present invention, each TALE repeat sequence of said core scaffold is made of 30 to 42 amino acids, more preferably 33 or 34 wherein two critical amino acids (the so-called
30 repeat variable dipeptide, RVD) located at positions 12 and 13 mediates the recognition of one nucleotide of said TALE binding site sequence; equivalent two critical amino acids can be located at positions other than 12 and 13 specially in TALE repeat sequence taller than 33 or 34 amino acids long. Preferably, RVDs associated with recognition of the different nucleotides are HD for

recognizing C, NG for recognizing T, NI for recognizing A, NN for recognizing G or A. In another embodiment, critical amino acids 12 and 13 can be mutated towards other amino acid residues in order to modulate their specificity towards nucleotides A, T, C and G and in particular to enhance this specificity. A TALE nucleic acid binding domain usually comprises between 8 and 30 TALE repeat sequences. More preferably, said core scaffold of the present invention comprises between 8 and 20 TALE repeat sequences; again more preferably 15 TALE repeat sequences. It can also comprise an additional single truncated TALE repeat sequence made of 20 amino acids located at the C-terminus of said set of TALE repeat sequences, i.e. an additional C-terminal half- TALE repeat sequence.

The invention is more particularly related to the following objects:

1. A method of engineering an immune cell for specifically targeting a cell comprising:

   (a) Providing an immune cell;

   (b) Engineering said immune cell to render said cell sensitive to at least two input signals such that the combination of input signals induces combination of at least two transmitter domains which results in activation of said immune cell, wherein each transmitter domain alone does not activate said immune cell.

2. The method of claim 1 wherein at least one said input signal is the recognition of a specific ligand by said immune cell which is engineered by expressing at the surface of the cell a chimeric antigen receptor (CAR) comprising an extracellular ligand binding domain capable of recognizing said specific ligand and an intracellular domain comprising a transmitter domain capable of activating said immune cell in combination with another transmitter domain optionally via a signaling protein.

3. The method of claim 1 wherein at least said two input signals are the recognition of different specific ligands by said immune cell which is engineered by expressing at the surface of the cell chimeric antigen receptors (CARs) comprising extracellular ligand binding domains capable of recognizing said different specific ligands and intracellular domains comprising transmitter domains capable of activating immune cell in combination.

4. The method according to any one of claims 1 to 3 wherein said input signal is an external stimulus selected from the group consisting of: the presence of particular small molecules, chemokines, cytokines, physicochemical conditions, hypoxia.

5. The method of claim 4 wherein external stimulus is hypoxia and wherein said immune cell is engineered to express at least one transmitter domain under the control of a hypoxia inducible promoter.

6. The method of claim 4 wherein external stimulus is hypoxia and wherein said immune cell is engineered to express at least one chimeric antigen receptor comprising a transmitter domain under the control of a hypoxia inducible promoter and wherein the recognition of a ligand of said chimeric antigen receptor is another input signal.

7. The method according to any one of claims 1 to 6 wherein said transmitter domains are a protease and a substrate protein comprising a signaling protein linked to a membrane anchoring domain via a protease cleavage site such that said protease cleavage induces the release of the signaling protein capable of activating said immune cell.

8. The method of claim 7 wherein said protease is selected from the group consisting of: TEV protease, Factor Xa, thrombin, engineered viral proteases.

9. The method according to any one of claims 1 to 8 wherein said transmitter domains are split proteins which assemble to reconstitute a signaling protein capable of activating said immune cell.

10. The method of claim 9 wherein said split proteins are split proteases which assemble together to reconstitute a protease capable of cleaving a substrate protein and releasing a signaling protein capable of activating said immune cell.

11. The method of claim 9 wherein said split proteins are split kinases which assemble together to reconstitute a kinase which activates a signaling protein capable of activating said immune cell.

12. The method of claim 9 wherein said split proteins are a split-inteins which assemble together to reconstitute an intein which excises the intein sequence and join with a peptide bond flanking signaling protein sequences capable of activating said immune cell.

13. The method according to any one of claims 1 to 8 wherein said transmitter domains are members of a signaling pathway which can recruit a scaffold protein.

14. The method of claim 13 wherein said scaffold protein is SYK tyrosine kinase or ZAP70.

15. The method according to any one of claims 1 to 8 wherein said transmitter domains are dimeric proteins.

16. The method according to any one of claims 1 to 8 wherein said transmitter domain is an autoinhibited compound.

17. The method according to any one of claims 1 to 8 wherein said engineered immune cell is initially inactivating for a gene and wherein said combination of transmitter domains is capable of complementing said inactivated gene.

18. A chimeric antigen receptor comprising an extracellular ligand binding domain and an intracellular domain which comprise at least one transmitter domain.

19. The chimeric antigen receptor of claim 18 wherein said transmitter domains are selected from the group consisting of: a protease, split protein, dimeric protein, member of a signaling pathway which can recruit a scaffold protein and autoinhibited compound.

20. A polynucleotide encoding a chimeric antigen receptor of claims 18 or 19.

21. An isolated immune cell which comprises a chimeric antigen receptor of claim 18 or 19.

22. An isolated immune cell obtained by any one the method according to any one of claims 1 to 17.

23. An isolated immune cell of claim 21 or 22 for its use as a medicament.

24. An isolated immune cell according to any of claims 21 to 23 for treating a cancer, an auto-immune condition or an infection by a pathogen.

25. A method of treating a subject in need thereof comprising:

    (a) Providing a immune cell according to claim 21 or 22;

    (b) Administrating said immune cells to said patient.

Examples

Example 1: Control of T cell activation through CAR-mediated regulation of CARMA1 protein.

The scaffold protein caspase recruitment domain-containing membrane-associated guanylate kinase protein-1 (CARMA1) is a member of the MAGUK family of kinases (Roche, Ramadas et al. 2013). CARMA1 constitutes a crucial signalosome for the transmission of the T cell Receptor (TCR) signaling and in general for T cell activation. The intracellular CARMA1 concentration is a key element in the regulation of its activity. An enhancement of the CARMA1 signaling has been observed at low and moderate concentrations while a decrease of the activity has been reported at high concentration dues to the sequestration of the different components away form each other (biphasic response). Following TCR engagement, CARMA1 recruits different proteins forming a multi –protein complexes that finally can activate two different signaling cascade: NF-κB and c-jun N-terminal kinase (JNK) (Blonska and Lin 2009).

CARMA1 is composed by five structural domains connected by linker regions. Among these five domains three constitute the membrane guanilate kinase domain (MAGUK): a PDZ homology domain (post synaptic density protein), a SRC homology domain (SH3) and a guanylate kinase domain (GUK). The MAGUK domain is necessary for cellular adhesion, formation of multi-domain complexes and signal transduction, thus this region is essential for CARMA1 regulating its localization at the membrane and its state of oligomerization. The N-terminal domain of CARMA1 is responsible for the activation and recruitment (CARD) of different proteins. Indeed the CARD domain is responsible for the interaction with B-cell CLL-lymphoma 10 (Bcl10) which *per se* mediates activation of NF-kB and JNK. The N-terminal domain is structurally followed by a coiled-coil domain which is responsible for the oligomerization state of CARMA1 and can regulate the binding of this last to the mucosa-associated lymphoma translocation gene1 (MALTA1) (essential for the activation of NF-kB). Finally the linker region between the coiled-coil domain and the MAGUK domain appears to play an important role in restraining the conformation of CARMA1 in a “closed” (inactive) form. On the contrary the phosphorylation of the serine residues of this zone produced by PKCθ (and other kinases see Table 1) promotes a high level of CARM1 activation with the following boost of the NF-κB signaling pathway. The negatively regulation of this pathway is operated by PP2A, which remove the phosporylation of CARMA1 at the specific residue S645.

The stimulation of TCR after recognizing the antigen is linked to the recruitment of CD28 which

leads to the activation of PKCθ, which in turn phosphorylates and activates CARMA1. Once activated CARMA1 binds to Bcl10 via CARD-CARD interactions; this binary complex recruits MALT1 to form a ternary complex: CARMA1- Bcl10- MALT1 (CBM). The CBM complex is necessary for the activation of the NF-κB and JNK. All the proteins reported in Table 1 have been characterized for their interactions with CARMA1 at different level and in different roles (see Figure 21).

Table 1: Proteins that Interact with the CARMA1 signalosome

KINASES	UBIQUITIN LIGASES	OTHER
PKCq	UBC13-UEV1A	Bcl10
IKK Complex	TRAF6	MALT1
PDK1	TRAF2	ADAP
CaMKII	cIAP2	Caspase 8
HPK1	NEDD4	Net1
CK1a	ITCH	
Akt	CBL-b	
TAK1	COP9	
RIP2	STUB1	
MKK7 (JNK activation)	CYLD (de-ubiquitylating enzyme)	
Calcineurin (phosphatase)	A20 (de-ubiquitylating enzyme)	
PP2A (phosphatase)		

Split-protein based systems

In a first example, the inventor plan to use one of the kinases listed in Table 2 as system of split proteins which once reconstituted (after co-localization of two CARs) will phosphorylate the serine residues on the linker region of the CARMA1 protein giving the start signal to the NF-κB and JNK. The generation of a split kinase has been already reported with success in the case of a thymidine kinase (Massoud, Paulmurugan et al. 2010).

Table 2. CARMA1 Phosphorylation Sites (Thome, Charton et al. 2010).

Human CARMA1	S109	T110	S551	S552	S555	S565	S608	S637	S645
Kinase	CaMKII	PKCq	HPK1	PKCθ	IKKβ	?	CK1α	PKCθ	PKCθ
Effect of Mutation									
NF-κB	↓	↓	↓	↓	↓	↓	↑	Normal	↓
JNK	?	↓	?	↓	↓	?	Normal	?	↓

Scaffolding protein systems

In addition to its carboxy-terminal MAGUK-like features, CARMA1 contains a CARD motif and a coiled coil domain that are functionally crucial. CARD motifs are protein–protein interaction domains that can mediate homotypic CARD/CARD interactions between two, or possibly even 5 three CARD containing binding partners. The CARD of CARMA1 mediates homotypic interaction with the adaptor Bcl10 which contains an amino-terminal CARD motif and a Ser/Thr-rich carboxyl terminus of unknown structure. Bcl10 constitutively forms a complex with MALT1 necessary for the activation of JNK and NF-κB (see Figure 21). Hence a fully reconstituted CARMA1 can be obtained after co-localization of two CARs once harboring the carboxy-terminal MAGUK-like 10 features and a second one the CARD motif and the coiled coil domain; the reconstitution of the CARMA1 will allow the assembly of Bcl10 and MALT1 with the consequent activation of the two endogenous pathways JNK and NF-κB.

Example 2: Generation of two types of LCKs to inhibit and to stimulate the signaling cascade.

15 LCK (NCBI Reference Sequence: NP_005347.3) is one of the first molecules to be activated following TCR engagement (Borger, Filby et al. 2013; Borger, Zamoyska et al. 2013; Brownlie and Zamoyska 2013). LCK is constitutively active in T cell maintaining a low level of phosphorylation of the ζ chain of CD3 associated to TCR. LCK binds to the cytoplasmatic domain of CD8 following the interaction between TCR and peptide-MHC, the co-receptor CD8 drives LCK in proximity of the ζ 20 chain of CD3 associated to TCR. The targets of LCK are the tyrosine residues on the ITAM of the TCR associated ζ chain of CD3 but also CD3δ chain, CD3ε chain and ZAP70. The phosphorylation of ZAP70 promotes a conformational change which actives its kinase activity leading to the phosphorylation of LAT (adaptor molecule linker for activation T cells). In turn Lat will recruit multiple downstream adaptors and signaling molecules.

25 LCK is positively regulates by phosphorylation of an active tyrosine (394 aa) in the catalytic centre which stabilizes an active conformation. On the contrary LCK is also negatively regulated by phosphorylation of a tyrosine in its C-terminal domain (505 aa). The activating tyrosine residue is auto phosphorylated by LCK and dephosphorylated by CD45 and other phosphatase (such as PTPN6, PTPN22). The negatively regulating tyrosine is phosphorylated by CSK and 30 dephosphorylated by CD45.

The possibility to create a mutant of LCK which will be not phophorylated on the C-terminal tyrosine (505 aa) gives the possibility to engineered LCK in a way to have a constitutively $LCK^{(+)}$ [Y505 -> X505 + Y394]. On the contrary we could create a constitutively negatively regulated $LCK^{(-)}$

if we mutate the Y394 to any other residues (Y394-> X394 and Y505). This mutation should avoid the phosphorylation of this position and the consequently it should create a LCK$^{(-)}$.

Thus we can plan a schema in which a first CAR will recognize an antigen of an healthy cell with an inhibitory domain which will stimulate the transcription of a form constitutively negatively regulated of LCK$^{(-)}$. This first CAR will be coupled with a second one which contains a co-stimulatory domains which will activate the transcription of LCK$^{(+)}$ form, producing an high level of activation of the T cell (Figure 20).

<u>Example 3: Use of environmental condition (hypoxia) to control HIF1α (a.a. 380-603) mcCAR fusion surface presentation - mRNA delivery</u>

The schematic functioning of the HIFα-system is depicted in Figure 22 at both conditions (normoxia and hypoxia). In Figure 23 is shown different CAR architectures (single-chain and multi-chain). In the following experiments, the multi-chain CAR conformation was used.

Constructs and mRNA preparation

All constructs originated from the pCLS24707 (SEQ ID NO: 1) which encode the α-chain (SEQ ID NO: 2), β-chain (SEQ ID NO: 3) and γ-chain (SEQ ID NO: 4) of the multichain CAR (mcCAR). The sequence coding for the amino acids 380 to 603 of the Hypoxia-inducible factor 1-alpha (HIF1 accession number Q16665, (SEQ ID NO: 5) was synthetized, in two parts, de novo (GeneCust) and cloned, using classical molecular biology technics, downstream the α-chain, using a short –GS-linker (SEQ ID NO: 6) leading to pCLS26580 (SEQ ID NO: 7).

All individual chains were amplified by PCR using oligo pairs α-chain-F/ α-chain-R, β-chain-F/ β-chain-R, γ-chain-F/ γ-chain-R and α-chain-F/ α-chain-HIF-R (SEQ ID NO: 8 to 9) prior to mRNA synthesis. mRNA encoding the α-chain, β-chain, γ-chain or α-chain-HIF1 were in vitro transcribed from the PCR product and polyadenylated using the mMessage mMachine T7 Ultra kit (Life technologies) following the manufacturer's instructions. RNAs were purified with RNeasy columns (Qiagen), eluted in cytoporation medium T and quantified by measuring absorbance at 260 nm

using a Nanodrop ND-1000 spectrophotometer. Quality of the RNA was verified on a denaturing formaldehyde/MOPS agarose gel.

Transfection

T lymphocytes were transfected by electrotransfer of messenger RNA using an AgilePulse MAX system (Harvard Apparatus) 3 to 6 days after activation. Following removal of activation beads, cells were pelleted, resuspended in cytoporation medium T at >28x106cells/ml. 5x106cells were mixed with 27.5 µg total RNA (10 µg α chain, 7.5 µg β chain and 10 µg γ chain) or with 32.5 µg total RNA (15 µg α chain-HIF1, 7.5 µg β chain and 10 µg γ chain) into a 0.4 cm cuvette. The electroporation consisted of two 0.1 ms pulses at 1200 V followed by four 0.2ms pulses at 130V. Following electroporation, cells were diluted into 2mL culture medium and incubated either at 37°C/ 5% CO_2 (referred as normoxia) or at 37°C with low O_2 concentration (referred as hypoxia) for 17h. Hypoxic conditions were created using an atmosphere generation system (2.5L AnaeroJAR assembly, Anaerogen 2.5L, Anaerobic indicator BR0055 Oxoid) as described by the manufacturer. A fraction of the cells from the hypoxia condition were kept and incubated at 37°C/ 5% CO_2 (normoxia) for 4-6 h.

Flow cytometry

First labelling for the detection of the α-chain was performed with anti-Fab'2-Biotin (goat anti-mouse IgG, Fab'2 fragment specific, 115-066-072, Jackson Immunoresearch) in PBS SVF2%, EDTA 2mM, azide 0.1% for 20 min at 4°C followed by a washing step with PBS SVF2% EDTA 2mM azide 0.1%. Second labelling was performed with Streptavidin-APC in PBS SVF2% EDTA 2mM azide 0.1% for 20 min at 4°C followed by a washing step in PBS. Cell viability was monitored using the efluor450 (ebioscience 65-0863-14) in PBS for 20min 4°C, followed by a washing step with PBS SVF2% EDTA 2mM azide 0.1%. Flow cytometry was performed using the MACSQUANT (Miltenyi Biotec) and data analysis was performed with the FlowJo software.

The data obtained clearly indicated an improved surface exposition in hypoxic condition (vs normoxia) when the α-chain was fused the HIF1α fragment (Figure 24).

Example 4: Use of environmental condition (hypoxia) to prevent cytotoxicity induced by HIF1α
5 **(a.a. 380-603) mcCAR fusion**

Transfection of T-cells was performed as in example 1 with 2 µg total RNA (0.94 µg α chain, 0.47 µg β chain and 0.62 µg γ chain). Surface detection was performed in normoxia and hypoxia as described in Example 3 (Figure 25A).

The cytolytic activity and specificity of engineered T-cells was assessed (1 day post transfection)
10 using a flow cytometry-based cytotoxicity assay in normoxia. In this assay target cells presenting the CAR target antigen (target+) and target cells not presenting the CAR target antigen (target-) are labelled with either CellTrace™ CFSE or CellTrace™ violet. The mixed target cell populations (1:1 ratio) was co-incubate at 37°C with various ratio of engineered effector CAR T cells (Effector/Target ratio of 10:1) in a final volume in X-Vivo-15 media, for a 4h time periods.
15 The whole cell population was recovered and labeled with eFluor780 viability marker before being fixed by 4% PFA. Fixed cells were analyzed by flow cytometry to determine their viability (target+, target- and effector T-cells). Flow cytometry and data analysis were performed as described in Example 3 (Figure 25B).

20 **Example 5: Use of environmental condition (hypoxia) to control HIF1α (a.a. 380-603) mcCAR fusion surface presentation and cytotoxicity – lentiviral delivery**

The alpha-HIF, beta, gamma chains were amplified by PCR, using oligo pairs GAα-chain-F/ GAα-chain-HIF-R, GAβ-chain-F/ GAβ-chain-R, GAγ-chain-F/ GAγ-chain-R respectively (SEQ ID NO: 15 to20). The three chains were assembled, using the Gibbson assembly protocol (New England
25 Biolabs) in a lentiviral plasmid under the control of an SFFV promoter leading to pCLS26949 (SEQ

ID NO: 21). Viral vectors were produced by GIGA-viral vectors (Belgium) from pCLS26949 (SEQ ID NO: 21) and pCLS24707 (SEQ ID NO: 1) encoding the alpha chain without the HIF domain.

Surface labelling

Following lentiviral transduction, cells were incubated at 37°C/ 5% CO_2 (referred as normoxia). 3 to 5 10 days post transduction, engineered T-cells were incubated either at 37°C/ 5% CO_2 (referred as normoxia) or at 37°C with low O_2 concentration (referred as hypoxia) for various time periods (1-24 hours). Hypoxic conditions were created as described by the manufacturers using either an atmosphere generation system (2.5L AnaeroJAR assembly, Anaerogen 2.5L, Anaerobic indicator BR0055 Oxoid) or the Oxyrase Enzyme System (EC-Oxyrase) or combination of the two methods. 10 Detection of surface presentation of the CAR was performed as described in Example 3.

Induced cytotoxicity

The cytolytic activity and specificity of engineered T-cells was assessed using a flow cytometry-based cytotoxicity assay in hypoxia or normoxia. In this assay target cells presenting the CAR target antigen (target+) and target cells not presenting the CAR target antigen (target-) are labelled with 15 either CellTrace™ CFSE or CellTrace™ violet. The mixed target cell populations (1:1 ratio) was co-incubate at 37°C with various ratio of engineered effector CAR T cells (Effector/Target ratio of 10:1 to 1:1) in a final volume in X-Vivo-15 media, for various time periods (4h to 24h).

The whole cell population was recovered and labeled with eFluor780 viability marker before being fixed by 4% PFA. Fixed cells were analyzed by flow cytometry to determine their viability (target+, 20 target- and effector T-cells). Flow cytometry and data analysis were performed as described in Example 3.

25

Example 6: Use of environmental condition (hypoxia) to control mcCAR surface presentation by alternative HIF1α or HIF3α domains

All constructs originated from the pCLS24707 (SEQ ID NO: 1) which encode the α-chain (SEQ ID NO: 2), β-chain (SEQ ID NO: 3) and γ-chain (SEQ ID NO: 4) of the multichain CAR (mcCAR). The sequence coding for the amino acids 344 to 417 (SEQ ID NO:22) or 530-652 (SEQ ID NO: 23) of the Hypoxia-inducible factor 1-alpha (HIF1 accession number Q16665) were assembled and cloned from de novo synthetized genes (GeneCust) as in Example 1, leading to pCLS26959 and pCLS26960 (SEQ ID NO: 24 to 25) respectively.

The sequence coding for the amino acids 480 to 571 (SEQ ID NO: 26) or 466-571 (SEQ ID NO: 27) of the Hypoxia-inducible factor 3-alpha (HIF3 accession number Q9Y2N7) were assembled and cloned from de novo synthetized genes (GeneCust) as in Example 3, leading to pCLS26961 and pCLS26962 (SEQ ID NO: 28 to 29) respectively.

The sequence coding for the amino acids 380 to 630 of the Hypoxia-inducible factor 1-alpha (HIF1 accession number Q16665, (SEQ ID NO: 5) was assembled and cloned from de novo synthetized genes (GeneCust), using classical molecular biology technics, downstream the α-chain, using a short –EA- linker (SEQ ID NO: 30) leading to pCLS26784 (SEQ ID NO:31).

Synthesis of mRNA, transfection, normoxia or hypoxia conditions, and flow cytometry in were generated and performed as described in Example 3.

The data obtained clearly indicated an improved surface exposition in hypoxic condition (vs normoxia) with the different fusions of HIF1α and HIF3α fragment to the alpha chain (Figure 26 A-F).

Example 7: Design of a dual receptors gate

In a general aspect, the system is composed of two membrane protein partners that are interacting upon co-localization (triggered by the binding to the two target antigens) and releasing a transmitter protein (Figure 27).

5 **Assembly of the membrane protein partners**

The first membrane protein partner is composed of different blocs (from the N- to the C-termini): (a) a signal sequence for the addressing to the membrane and an antigen-specific targeting regions (SEQ ID NO: 32 to 38), (b) an extracellular spacer domain (so-called hinge) (SEQ ID NO: 39 to 41), (c) a transmembrane domain (SEQ ID NO: 42 to 46), and (d) an intracellular structural
10 and/or signaling linker domain (SEQ ID NO: 47 to 70) and (e1) one of the interacting partner domains (SEQ ID NO: 71 to 77) (Figure 28).

The second membrane protein partner is composed of different blocs (from the N- to the C-termini): (a) a signal sequence for the addressing to the membrane and an antigen-specific targeting regions (SEQ ID NO: 32 to 38), (b) an extracellular spacer domain (so-called hinge) (SEQ
15 ID NO: 39 to 41), (c) a transmembrane domain (SEQ ID NO: 42 to 46), (d) an intracellular structural and/or signaling linker domain (SEQ ID NO: 47 to 70), (e2) the second interacting partner domain (SEQ ID NO: 78 to 79), (f) a transcription factor composed of a DNA binding domain (SEQ ID NO: 80 to 81) and (g) a transactivation domain (SEQ ID NO: 82 to 82) (Figure28). The 2 parts of the split-ubiquitin system is the Nub/Cub in human is depicted by SEQ ID NO:72-77 and NO:79 respectively.
20 The 2 interactor domains TEV of protease system from Tobacco Etch virus are depicted by SEQ ID NO:71 and 78.

The blocs are designed to incorporate at each extremity a type IIs restriction sites (BbsI) that allows enzymatic creation of unique overhangs for each position:

For the first membrane protein partner: atgg-(a)-tccc; tccc-(b)-gata; gata-(c)caga; caga-(d)-gagc;
25 gagc-(e1)-gaat (Figure 29).

For the second membrane protein partner: atgg-(a)-tccc; tccc-(b)-gata; gata-(c)caga; caga-(d)-gagc; gagc-(e2)-tgga; tgga-(f)-tagc; tagc-(g)-gaat (Figure29).

The different blocs are either synthetized de novo (GeneCust), assembled from oligos or amplified by PCR and inserted in either a pUC57 or pJET cloning vectors using classical molecular biology technics. Inserts containing the bloc of interest are amplified from the pUC57 or pJET by PCR with oligonucleotides located a few dozen base pairs upstream and downstream the cloned position (SEQ ID NO: 86 to 89). The PCR products are gel-purified and diluted to 1/40 of their size in base pairs (ng/µl). The sequences coding for the membrane protein partners are assembled in a one-pot reaction by iterative rounds of restriction and ligation (1 cycle: 37°C, 5 min, 45 cycles: 2 min at 37°C, 5 min at 16°C, 1 cycle : 5 min at 37°C, 1 cycle: 10 min at 80°C and 1 cycle: 2 min at 25°C) in presence of an pre-digested receiving plasmid (2 µl of each bloc, 1 µl receiving vector at 10ng/µl, 1 µl ATP at 50mM, 1 µl BbsI NEB, 1 µL T4 Ligase (5U/µl), 2 µL T4 Ligase Buffer 10x, total volume 20µl) (SEQ ID NO: 90) containing a kanamycin resistance gene marker. Examples of assembled membrane protein partners are given (SEQ ID NO: 92 to 147).

mRNA preparation

The sequence coding for the assembled membrane protein partners are sub-cloned (NcoI and HindIII) in a plasmid under the control of a T7 promoter (SEQ ID NO: 202) using classical molecular biology technics. Alternatively, the sequence coding for the assembled membrane protein partners are amplified by PCR using oligonucleotides pairs bringing a T7 promoter (SEQ ID NO: 149 to 151) using classical molecular biology technics. Additionally, for the mcCAR-based membrane protein partner, the beta and gamma chains are amplified from pCLS24707 (SEQ ID NO: 152) using oligonucleotides β-chain-F/ β-chain-R and γ-chain-F/ γ-chain-R (SEQ ID NO: 153 to 156).

mRNA encoding the membrane protein partners are in vitro transcribed from the PCR product and polyadenylated using the mMessage mMachine T7 Ultra kit (Life technologies) following the manufacturer's instructions. RNAs are purified with RNeasy columns (Qiagen), eluted in

cytoporation medium T and quantified by measuring absorbance at 260 nm using a Nanodrop ND-1000 spectrophotometer. Quality of the RNA is verified on a denaturing formaldehyde/MOPS agarose gel.

Transfection

5 T lymphocytes are transfected by electrotransfer of messenger RNA using an AgilePulse MAX system (Harvard Apparatus) 3 to 6 days after activation. Following removal of activation beads, cells are pelleted, resuspended in cytoporation medium T at >28x106cells/ml. 5x106cells are mixed with 1 to 30 µg total RNA into a 0.4 cm cuvette. The electroporation consisted of two 0.1 ms pulses at 1200 V followed by four 0.2ms pulses at 130V. Following electroporation, cells are 10 diluted into 2ml culture medium and incubated either at 37°C/ 5% CO_2.

Flow cytometry

First labelling for the detection of the membrane protein partners is performed with anti-Fab'2-Biotin conjugated (goat anti-mouse IgG, Fab'2 fragment specific, 115-066-072, Jackson Immunoresearch) in PBS SVF2%, EDTA 2mM, azide 0.1% for 20 min at 4°C followed by a washing 15 step with PBS SVF2% EDTA 2mM azide 0.1%. Second labelling is performed with Streptavidin-APC in PBS SVF2% EDTA 2mM azide 0.1% for 20 min at 4°C followed by a washing step in PBS. Cell viability is monitored using the efluor450 (ebioscience 65-0863-14) in PBS for 20min 4°C, followed by a washing step with PBS SVF2% EDTA 2mM azide 0.1%. Flow cytometry is performed using the MACSQUANT (Miltenyi Biotec) and data analysis is performed with the FlowJo software.

20 Examples of surface exposition of different membrane protein partners (SEQ ID NO: 96, 106, 110, 125, 126, 128, 129 and 131) are given in Figure 30.

Example 8: Dual receptors gate readouts – lentiviral delivery

To demonstrate the possibilities of the dual membrane protein partners' strategy, readouts based 25 on the expression of a reporter gene are built. These reporter systems are composed of several

repeats of either the TetO (7x) or the Gal4 (5x) operator sequence that are placed upstream of a minimal CMV promoter, allowing expression of an RQR8 or a renilla reporter gene placed downstream of this artificial promoter leading to pCLS26301, pCLS26303, pCLS27049 and pCLS27050 (SEQ ID NO: 157 to 160). These construct are cloned in a lentiviral expression vector. 5 Viral vectors are prepared using the commercially available lentiviral expression systems according to the manufacturer protocols.

To evaluate the possibility to monitor the expression of the RQR8 gene, transactivators composed of a DNA binding domain (TetO or Gal4) and a transcription activation domain (VP64 or NF-kB) are constructed (SEQ ID NO: 161 to 164). Corresponding mRNAs are produced as described in Example 10 7 and T-cells previously transduced with the reporter systems (readouts) are transfected with these mRNAs coding for the transactivators.

The data obtained clearly indicated the expression of the lentiviral delivered RQR8 cassette by mRNA transfection of the adequate transactivator (Figure 31).

The membrane protein partners are sub-cloned in a lentiviral plasmid under the control of an SFFV 15 promoter (SEQ ID NO: 165). Alternatively, the assembled membrane protein partners are sub-cloned in a lentiviral production plasmid (under the control of an SFFV promoter (SEQ ID NO: 165)) upstream a 2A cis-acting hydrolase element followed by a reporter marker (e.g. fluorescent proteins). Standard molecular biology technics such as PCR, enzymatic restriction digestion and ligation are applied to create all constructions. Viral vectors are either obtained from commercial 20 providers or prepared using commercially available lentiviral expression systems according to the manufacturer protocols.

The two interacting membrane protein partners are then delivered in T-cell previously transduced with the reporter systems (readouts) as either mRNA (Example 7) or lentiviral vectors or combination of the two. Expression of the reporter system is recorded in presence of target cells

presenting antigens for (i) both interacting membrane protein partners, (ii) only one interacting membrane protein partner and (iii) none of the interacting membrane protein partners.

Example 9: Knock-out of proteins involved in the TCR signaling pathway

5 To create a T-cell custom readout system for the dual membrane protein partners strategy, knock-outs of genes coding for proteins involved in the TCR pathway (SEQ ID NO: 166 to 174) are realized using TALEN (SEQ ID NO: 175 to 192). mRNA preparation and transfection is performed as described in Example 7. TALEN activity in T-cells is monitored at the endogenous locus using the enzymatic T7 assay using conventional protocols. The data obtained clearly indicated a high level 10 of targeted mutagenesis at all targeted loci using the designed TALEN (Figure 32).

The effect of the knock-out on the induced degranulation capacity of the engineered T-cell is assessed. Engineered T-cells are cultured in 96-well plates (80,000 cells/well) in a final volume of 100µl of X-Vivo™-15 medium (Lonza) for 6 hours at 37°C with 5% CO_2. Cell stimulation is performed with either Human T-Activator CD3/CD28 beads (Life Technologies, # 11132D) or PMA 15 (20ng/ml) and ionomycin (1uM) or PHA (1.5µg/mL). CD107a staining was done during cell stimulation, by the addition of an APC-conjugated anti-CD107a antibody (BD Biosciences) together with 1µg/ml of anti-CD49d (BD Biosciences), 1µg/ml of anti-CD28 (Miltenyi), and 1x Monensin solution (eBioscience). After the 6h incubation period, cells were stained with a fixable viability dye (eBioscience) and PE-conjugated anti-CD8 (Miltenyi) and analyzed by flow cytometry. The data 20 obtained clearly indicated a strong staining decrease for the knock-out engineered T-cell relative to WT T-cells (Figure 33).

Example 10: Complementation of knock-outs using the membrane protein partners strategy

The gene coding for the KO protein (e.g. ZAP70) (SEQ ID NO: 193) are cloned in the readout 25 systems described in Example 8 in place of the RQR8 or renilla genes. Alternatively, target DNA

sequences (SEQ ID NO: 247) of human transcription factors (e.g.: HNF1B and HNF1A) (SEQ ID NO: 195 and 196) are cloned to replace the TetO or the Gal4 operator sequence in the readouts. DNA sequences coding for these human transcription factors (e.g.: HNF1B and HNF1A) are synthetized de novo to create blocs (SEQ ID NO: 197 to 198) compatible with the assembly process of
5 membrane protein partners described in Example 7. Design of TALEN used to perform knock-out of genes (e.g. ZAP70), lentiviral vector production, mRNA preparation, T-cell transfection or transduction of the readouts and membrane protein partners is done as described in Examples 7, 8 and 9. The complementation of the knock-out is monitored using either the degranulation assay or a flow-based cytotoxicity assay in presence of target cells presenting antigens for (i) both
10 interacting membrane protein partners, (ii) only one interacting membrane protein partner and (iii) none of the interacting membrane protein partners.

Example 11: Design of a bispecifc CAR (biCAR) gate

Assembly of the biCAR partners

15 The biCAR partners partner are composed of different blocs (from the N- to the C-termini): (a) a signal sequence for the addressing to the membrane and an antigen-specific targeting domain (b) an extracellular spacer domain (so-called hinge), (c) a transmembrane domain and (d) an intracellular activation and/or costimulatory domain (Figure 23).

The functioning of such biCAR gates is shown in Figure

20 Antigen-specific targeting domains are selected from pools of candidates either based on biochemical criteria (e.g. equilibrium dissociation constants (K_D), on- and off- rates (k_{off} and k_{on}) or randomly as collections or libraries.

The biCARs are either synthetized de novo or assembled as in previous examples. mRNA preparation, transfection and flow cytometry experiments are performed as in previous examples
25 according to the manufacturer recommendations.

The biCAR partners are sub-cloned in a lentiviral plasmid either under the control of the adequate promoter or under the adequate promoter upstream a 2A cis-acting hydrolase element followed by a different reporter marker (e.g. fluorescent proteins) for each biCAR partners (one reporter marker per library). Standard molecular biology technics such as PCR, enzymatic restriction 5 digestion and ligation are applied to create all constructions.

Viral vectors of individual biCARs, collection of biCARs or libraries of biCARs are either obtained from commercial providers or prepared using commercially available lentiviral expression systems according to the manufacturer protocols.

10 **Example 12. Characterization of biCAR gate system in immortalized or in primary T-cells**

Both biCAR partners composing the biCAR gate are delivered in immortalized human T-cells (Jurkat) or in primary T-cells as lentiviral vectors either individually or as libraries.

The transduced T-cells are purified for double positive surface biCAR expression or double positive reporter marker expression using bulk FACS sorting or magnetic separation.

15 The whole bulk double positive biCAR transduced population is then assessed for target cell driven activation (degranulation) using (i) a model cell line expressing only the 1st CAR target antigens and (ii) a model cell line expressing only the 2nd CAR target antigen. Populations that present no or weak activation induced by targets cells presenting only one antigen are isolated bulk using FACS sorting or magnetic separation.

20 These populations are then assessed for target cell driven activation (degranulation) using a model cell line expressing both CAR target antigens. Populations that present medium or strong activation induced by targets cells presenting both CAR antigens are isolated bulk using FACS sorting or magnetic separation.

The identity of both CARs is then confirmed by sequencing (or deep sequencing in case of 25 libraries) using standard molecular biology procedure.

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CLAIMS

1. A chimeric antigen receptor (CAR), which comprises at least:
   - an extracellular ligand binding domain capable of recognizing a specific ligand expressed at the surface of a tumor cell;
   - a transmembane domain;
   - an intracellular domain comprising at least an activation domain; and
   - an oxygen-sensitive polypeptide domain.

2. A CAR according to claim 1, wherein said oxygen-sensitive domain is selected between HIF1 alpha or HIF3 alpha, or have an identity of over 80%, preferably 90% or more preferably 95% with respectively SEQ ID NO:22, 23, 85, or SEQ ID NO:26, 27.

3. A CAR according to claim 1 or 2, wherein said extracellular domain further comprises a hinge.

4. A CAR according to claim 3, wherein said hinge is selected from CD8a, IgG1 or EpoR-D2, or have an identity of over 80%, preferably 90% or more preferably 95% with respectively SEQ ID NO:39, 40 or 41.

5. A CAR according to any one of claims 1 to 4, wherein said extracellular binding domain comprises a scFv directed to an epitope of EGFRvIII, CS1, CT83, GD3, MSCP, CD19, 5T4, ROR1, CD123 or CD33 cell target antigens.

6. A CAR according to according to any one of claims 1 to 5, wherein said extracellular binding domain comprises a scFv having comprising a polypeptide having an identity of over 80%, preferably 90% or more preferably 95% with SEQ ID NO: 32, 35, 38; SEQ ID NO: 33 ; SEQ ID NO:34 ; SEQ ID NO:36 or SEQ ID NO:37.

7. A CAR according to any one of claims 1 to 6,, wherein said transmembrane domain is selected from CD8a, 4-1BB, DAP10, CD28 or FceRIalpha, and have an identity of over 80%,

preferably 90% or more preferably 95% with respectively SEQ ID NO:42, 43, 44, 45 and 46.

8. A CAR according to to any one of claims 1 to 7, wherein said intracellular domain comprises a linker selected amongst CD3zeta, FceRIg, CD28, 4-1BB, OX40, DAP10, CD28, CD275, HVEM, LIGHT, CD40L, GITR, TIM1, SLAM, CD2, TLT-2, LAG3, DAP12, CD84, CD244, CD229, LTBR and CD278, or having an identity of over 80%, preferably 90% or more preferably 95% with respectively SEQ ID NO:47 to 70.

9. A CAR according to any one of claims 1 to 8, wherein said activation domain is CD3zeta.

10. A CAR according to any one of claims 1 to 9, wherein it further comprises a co-stimulation domain, such as that from 4-1BB or CD28.

11. A CAR according to anyone of claims 1 to 10, wherein the chimeric antigen receptor (CAR) is a single-chain CAR.

12. A CAR according to anyone of claims 1 to 10, wherein the chimeric antigen receptor (CAR) is a multi-chain CAR.

13. A CAR according to claim 12, wherein said multi-chain CAR comprises part of the α, β and γ chains from Fc receptor having respectively an identity of over 80%, preferably 90% identity or more preferably 95% identity with SEQ ID NO. 7, 3 and 4.

14. A CAR according to claim 13, wherein:

- the alpha-chain comprises extracellularly the CD8 hinge, FcRα as transmembrane domain, and intracellularly a part of FcRα combined with HIF1alpha or HIF3 alpha subunit;
- the beta-chain comprises the FcRβ as extracellular and transmembrane domain, and ΔITAM-41BB as intracellular co-stimulation domain;
- the gamma-chain comprises the FcRγ as transmembrane domain, and ΔITAM-CD3ζ as intracellular activation domain.

15. A method of engineering an immune cell for specifically targeting a cell, said method comprising:

(a) Providing an immune cell;

(b) Engineering said immune cell to render said cell sensitive to at least two input signals such that the combination of said input signals induces combination of at least a first and second transmitter domains, said combination resulting into a signal of activation of said immune cell, wherein:

- the first input signal is hypoxia

- the second input signal results from the recognition of a specific ligand by a chimeric antigen receptor (CAR) expressed at the surface of the immune cell comprising an extracellular ligand binding domain capable of recognizing said specific ligand and an intracellular domain comprising said transmitter domain capable of activating said immune cell in combination with the other transmitter domain.

- each transmitter domain alone does not activate said immune cell(c) expanding said engineered immune cell;

and

(c) expanding said engineered immune cells.

16. The method according to claim 15, wherein said transmitter domain becomes sensitive to hypoxia by being under the control of a hypoxia inducible promoter.

17. The method according to claim 15 or 16, wherein the sensitivity to said hypoxia condition is triggered by the alpha hypoxia inducible factor 1 (HIF-1α) or by the alpha hypoxia inducible factor 3 (HIF-3α).

18. The method according to claim 17, wherein the HIF-1α polypeptide sequence has over 80% identity, preferably 90% identity or more preferably 95% identity with SEQ ID NO. 5 or to SEQ ID NO.22-23, or the HIF-3α polypeptide sequence has over 80% identity, preferably 90% identity or more preferably 95% identity with SEQ ID NO. 26-27.

19. The method according to any one of claims 15 to 18, wherein said CAR is according to any one of claims 1 to 14.

20. An immune cell obtainable by the method of any one of claims 15 to 19.

21. An immune cell obtainable by any one of claim 1 to 8 for use as a therapeutic composition.

22. An isolated immune cell according to any of claims 1 to 11 for treating a cancer.

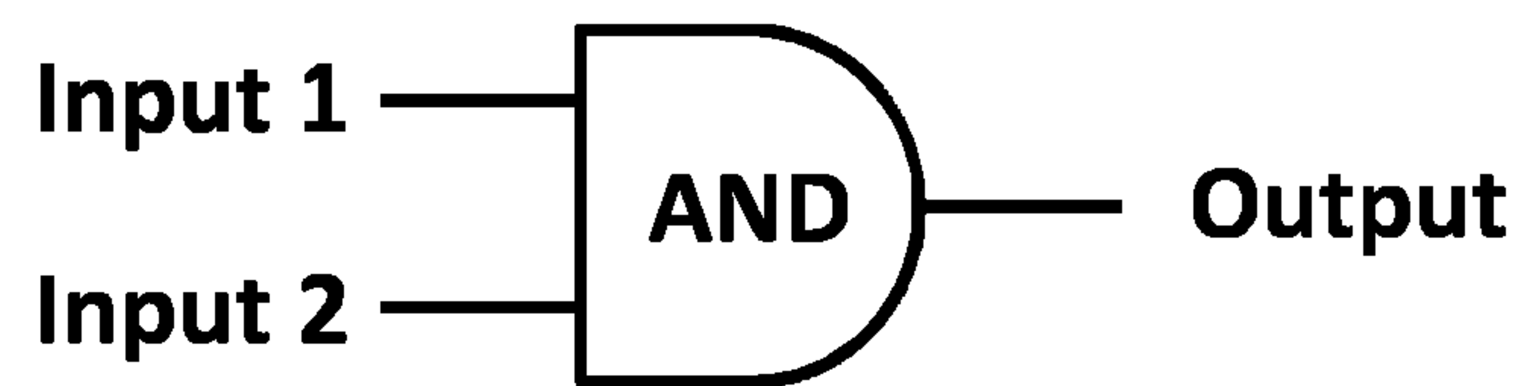
23. An isolated immune cell according to claim 12 for treating solid tumors.

24. A method for treating a subject in need thereof comprising:

(a) Providing an immune cell according to anyone of claims 20 to 23;

(b) Administrating said immune cells to said patient.

AND logic gate



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0	1	0
1	1	1

Fig. 1

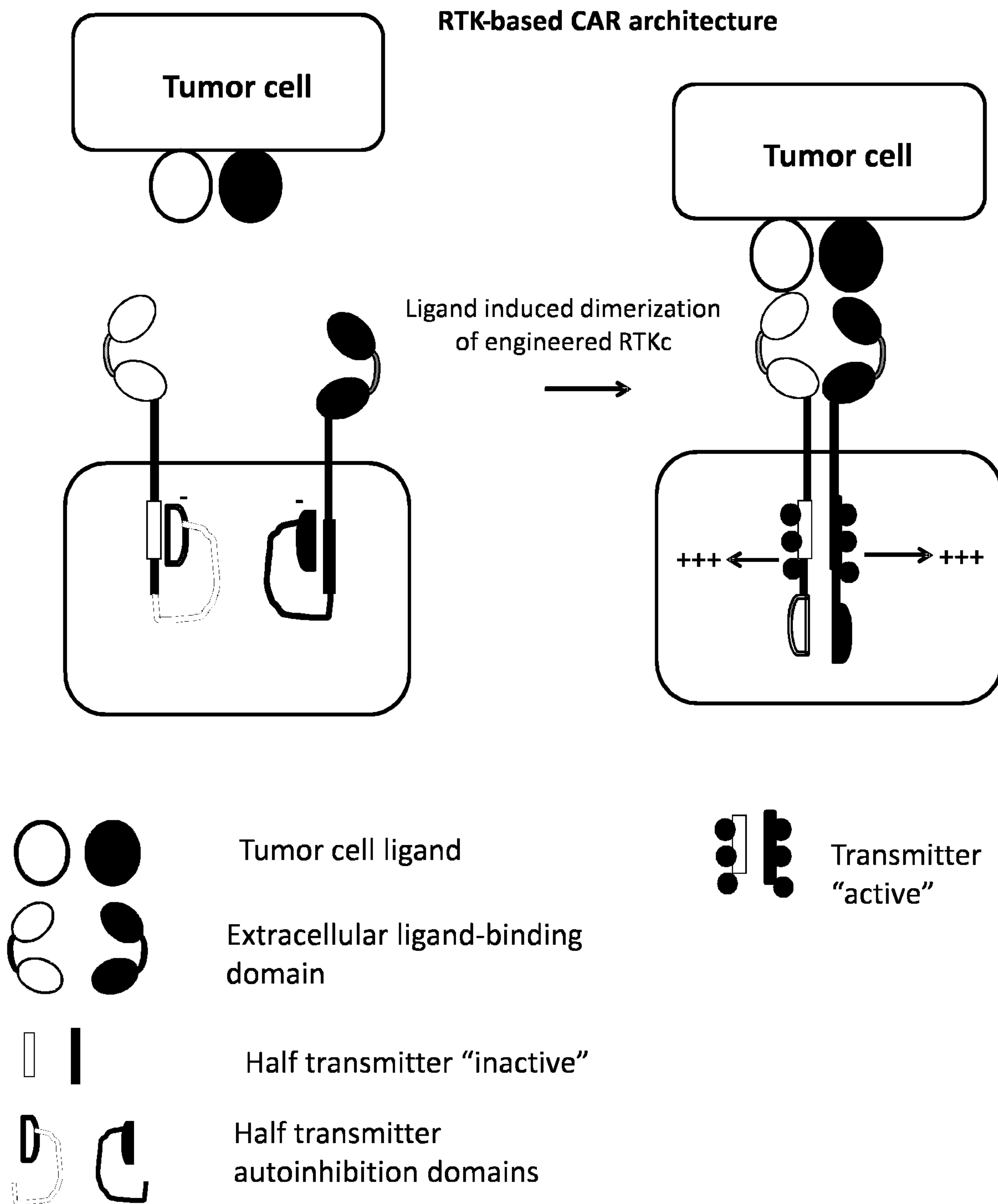
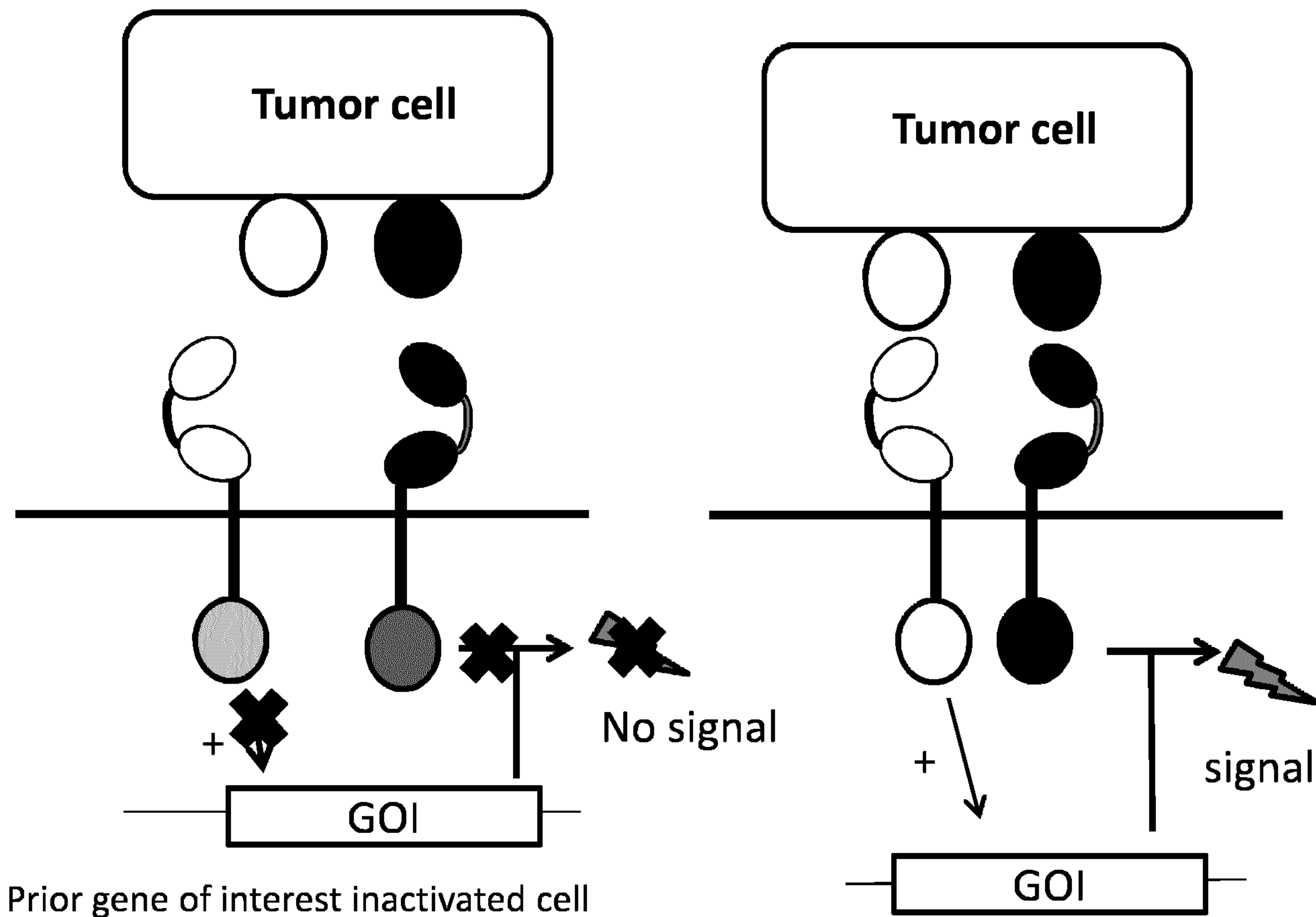


Fig. 2



Tumor cell ligand

extracellular ligand-binding domain

Transmitter domains

GOI

Gene of interest

Fig. 3

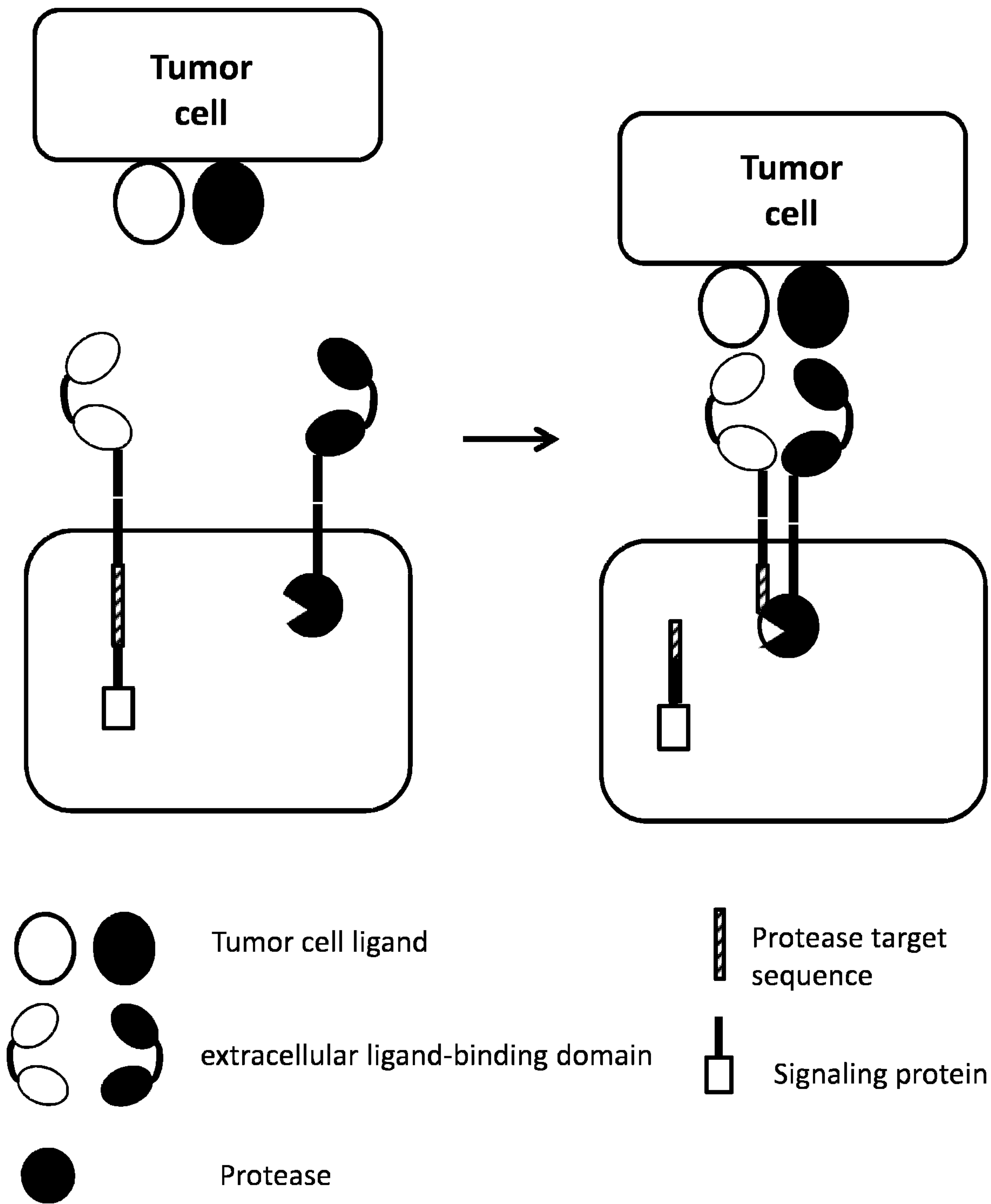


Fig. 4

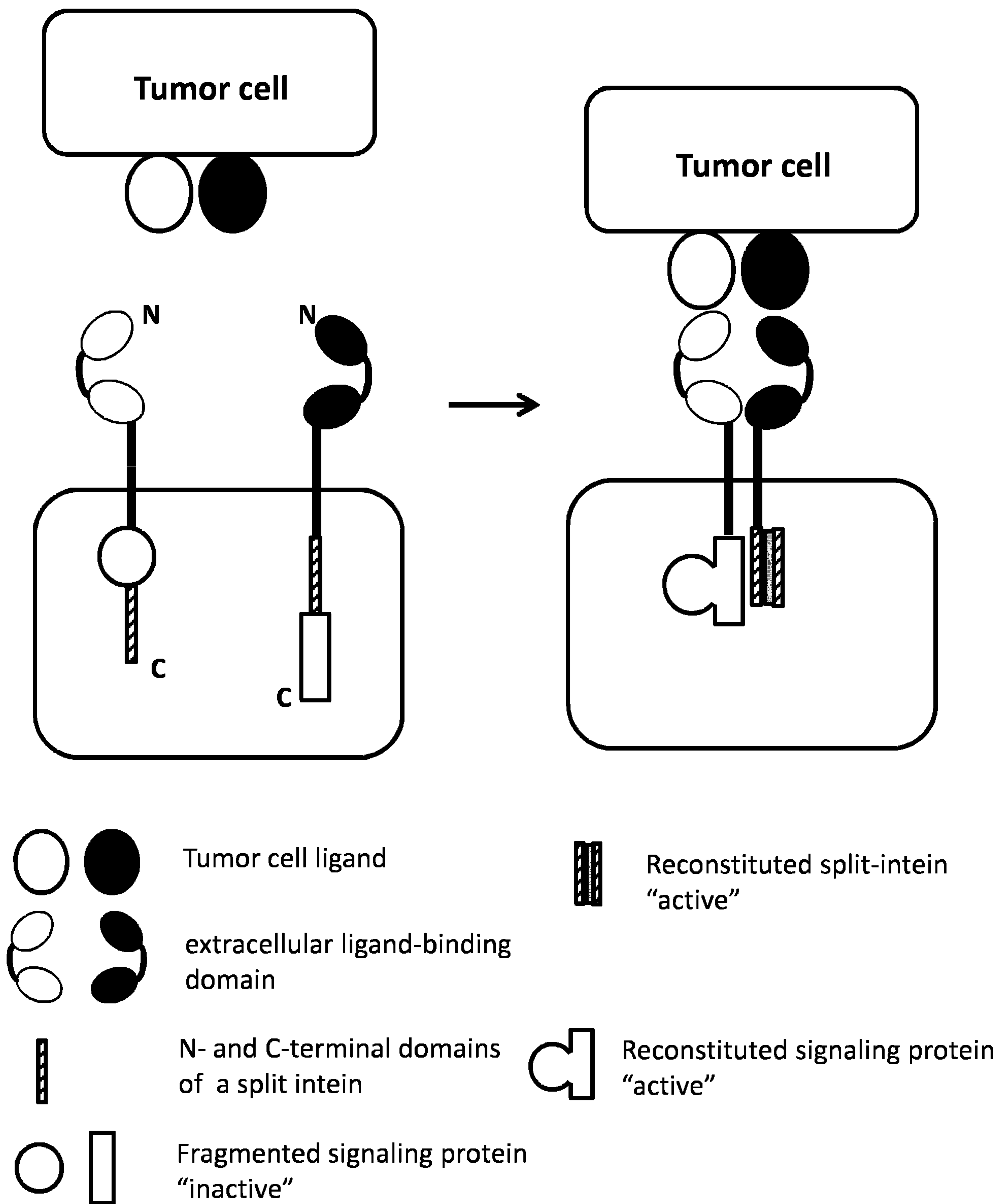


Fig. 5

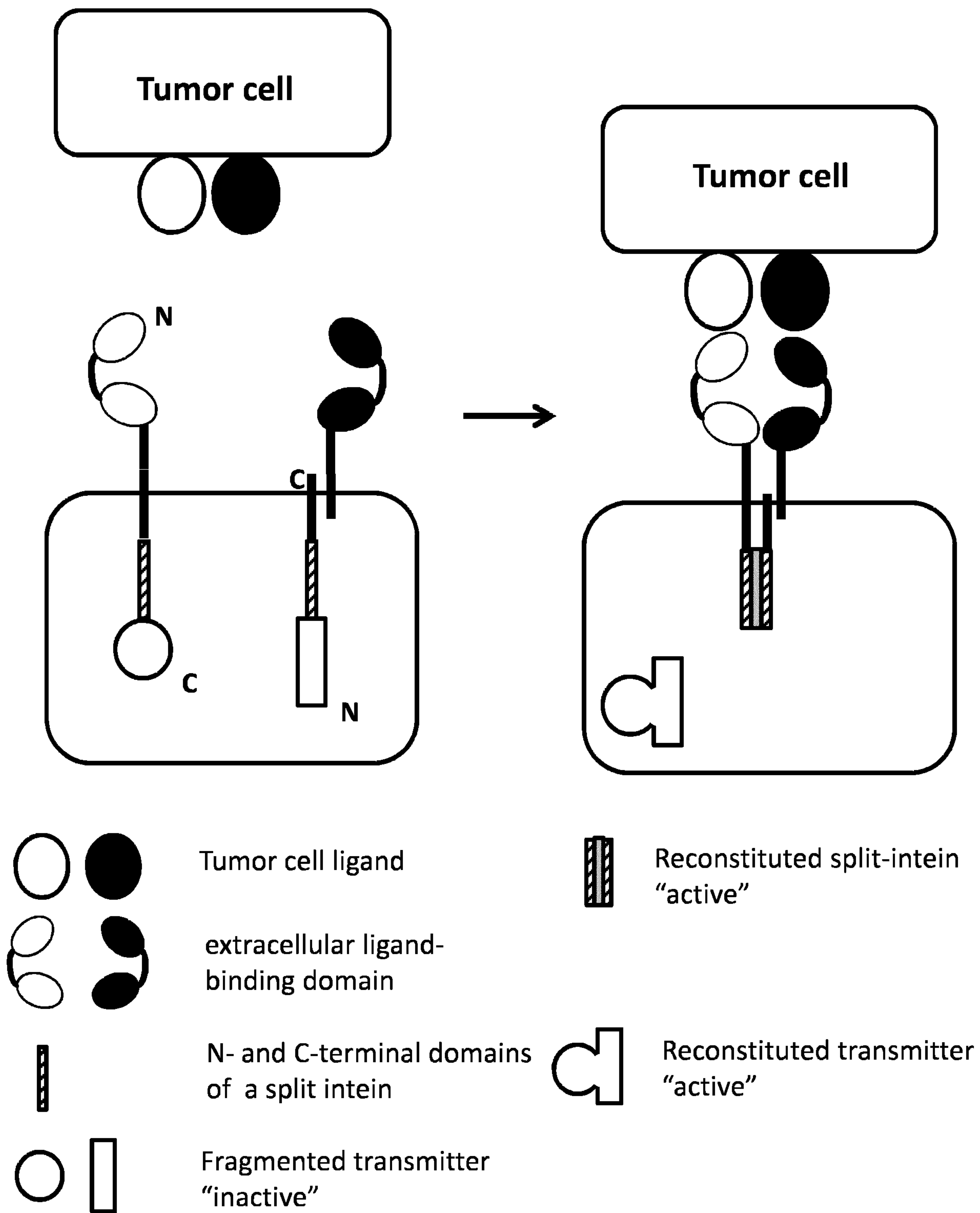


Fig. 6

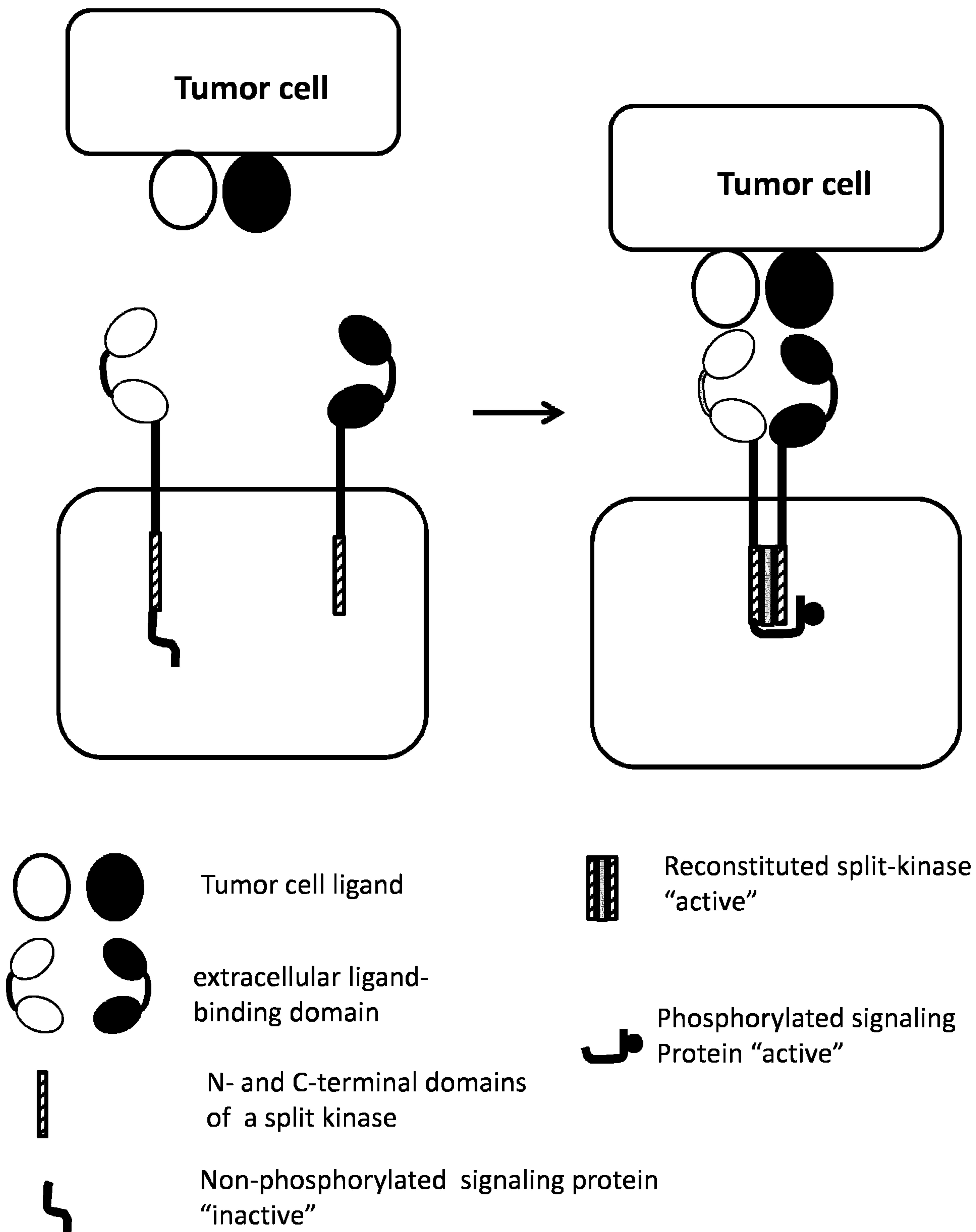


Fig. 7

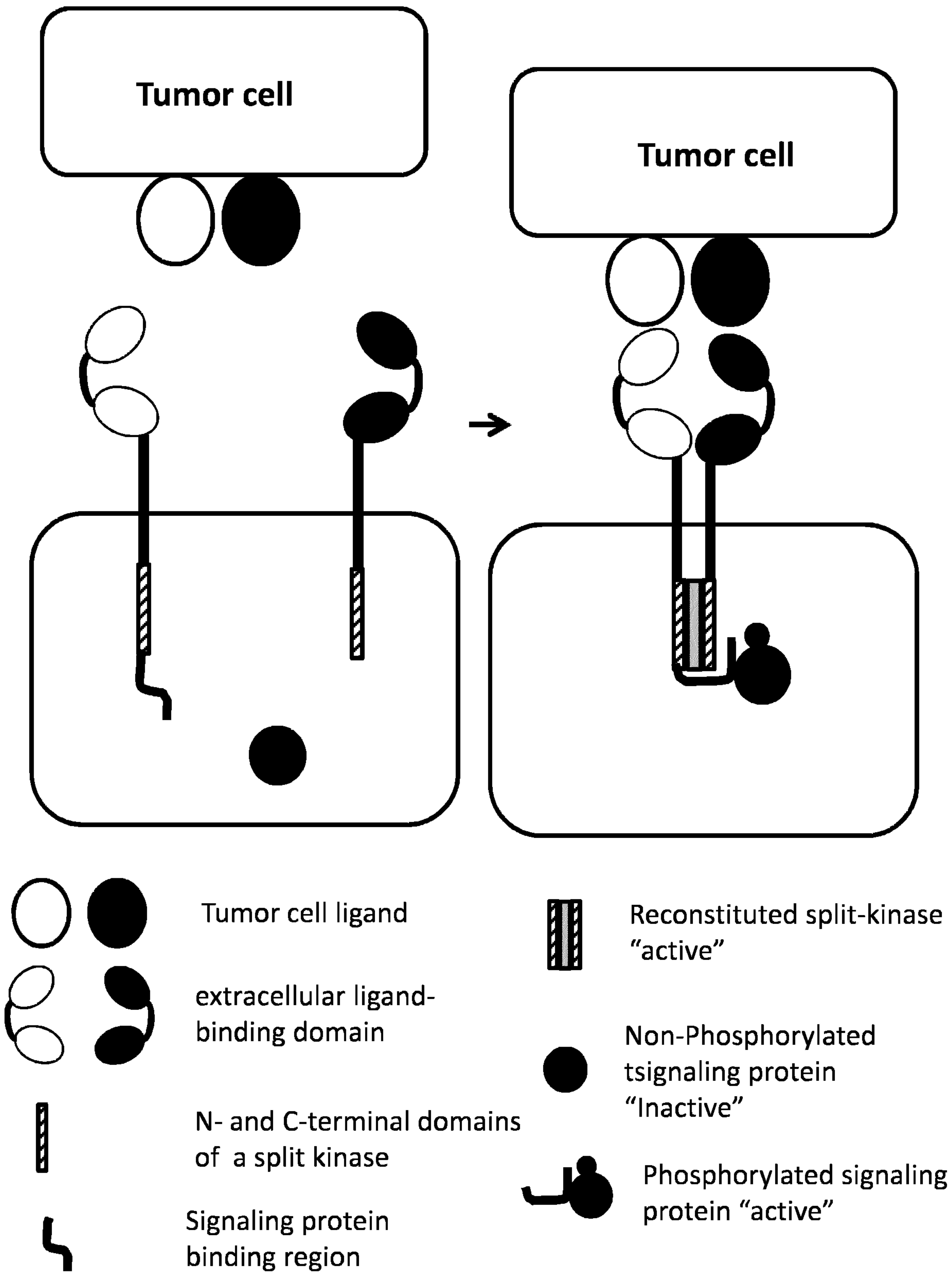
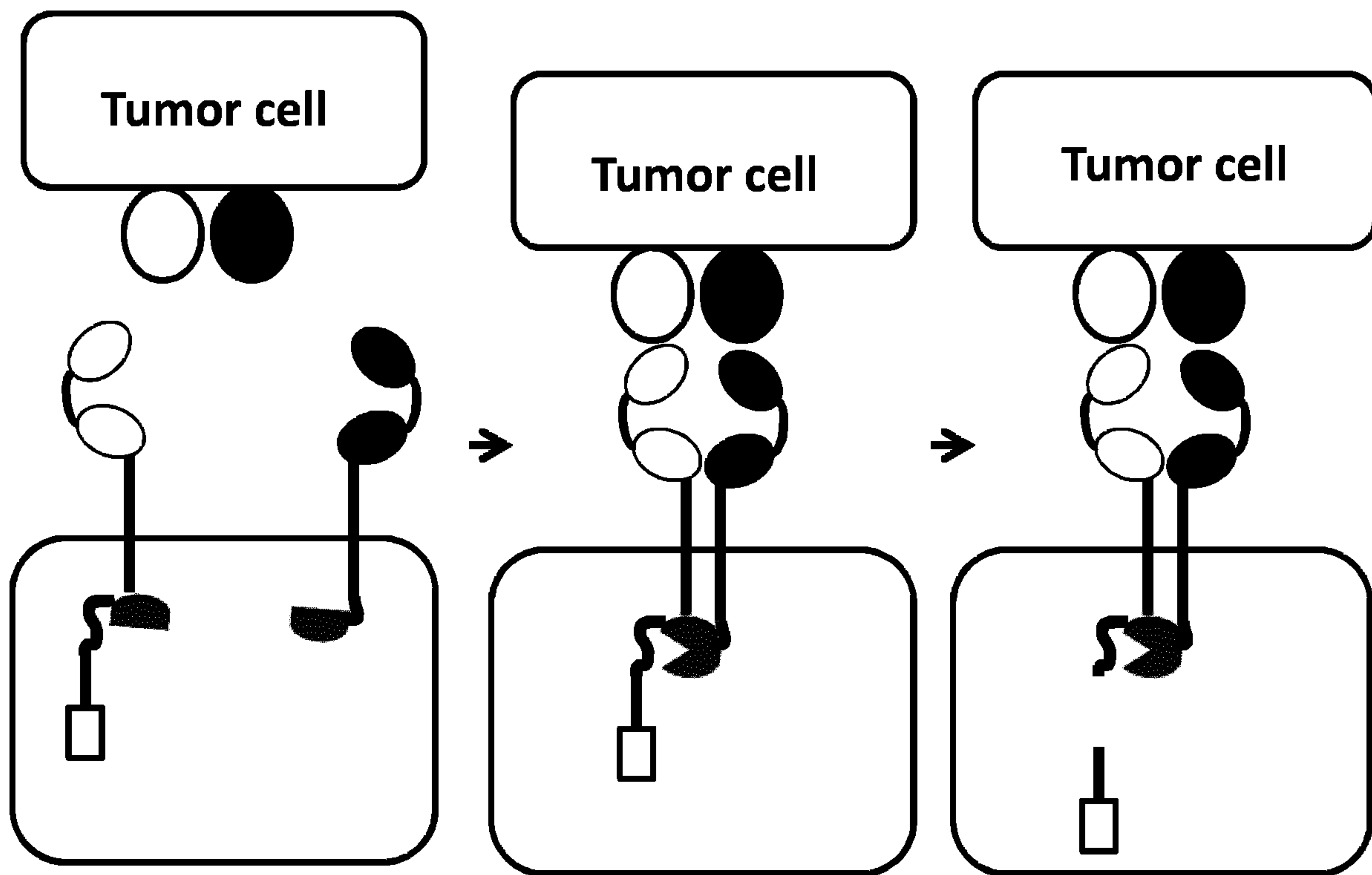


Fig. 8



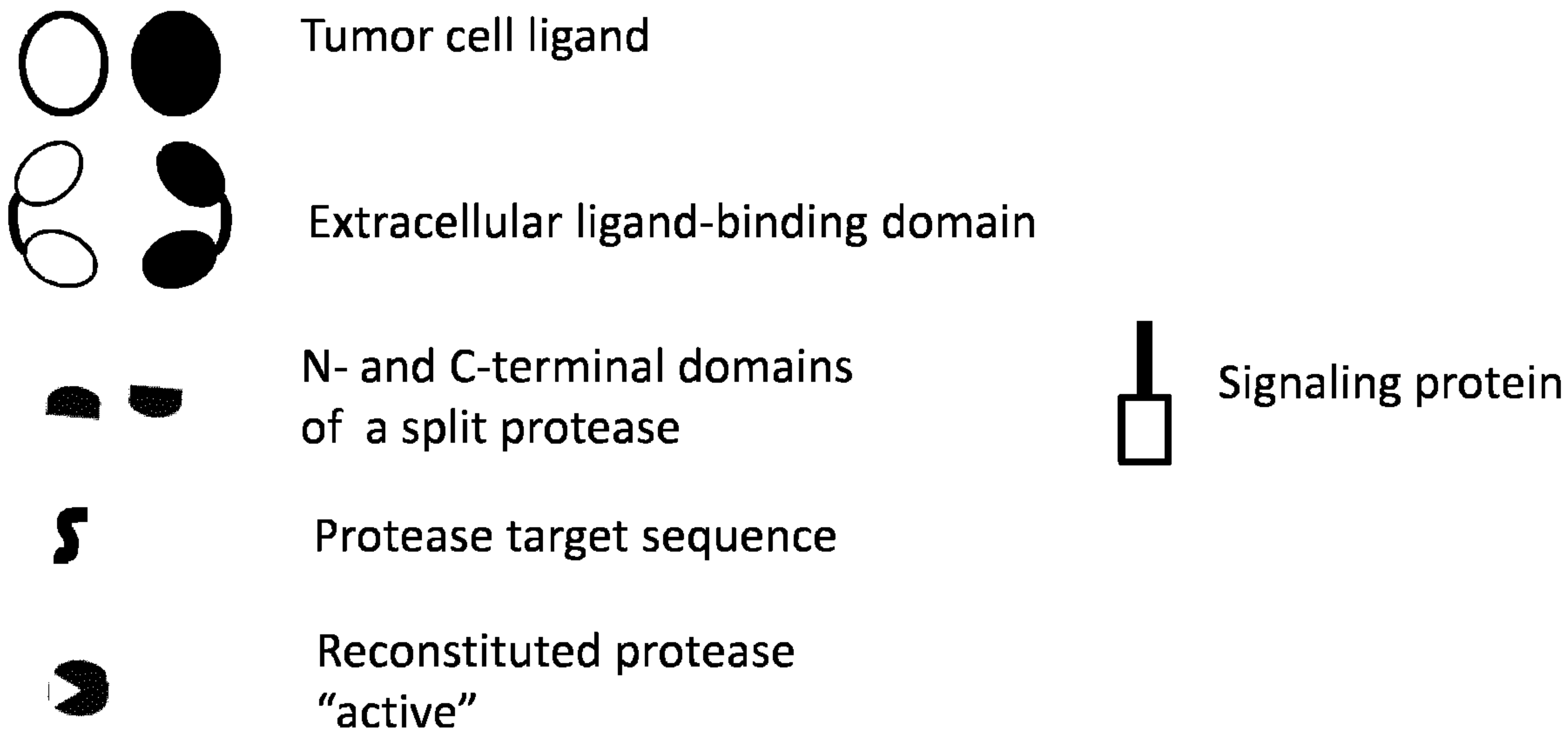


Fig. 9

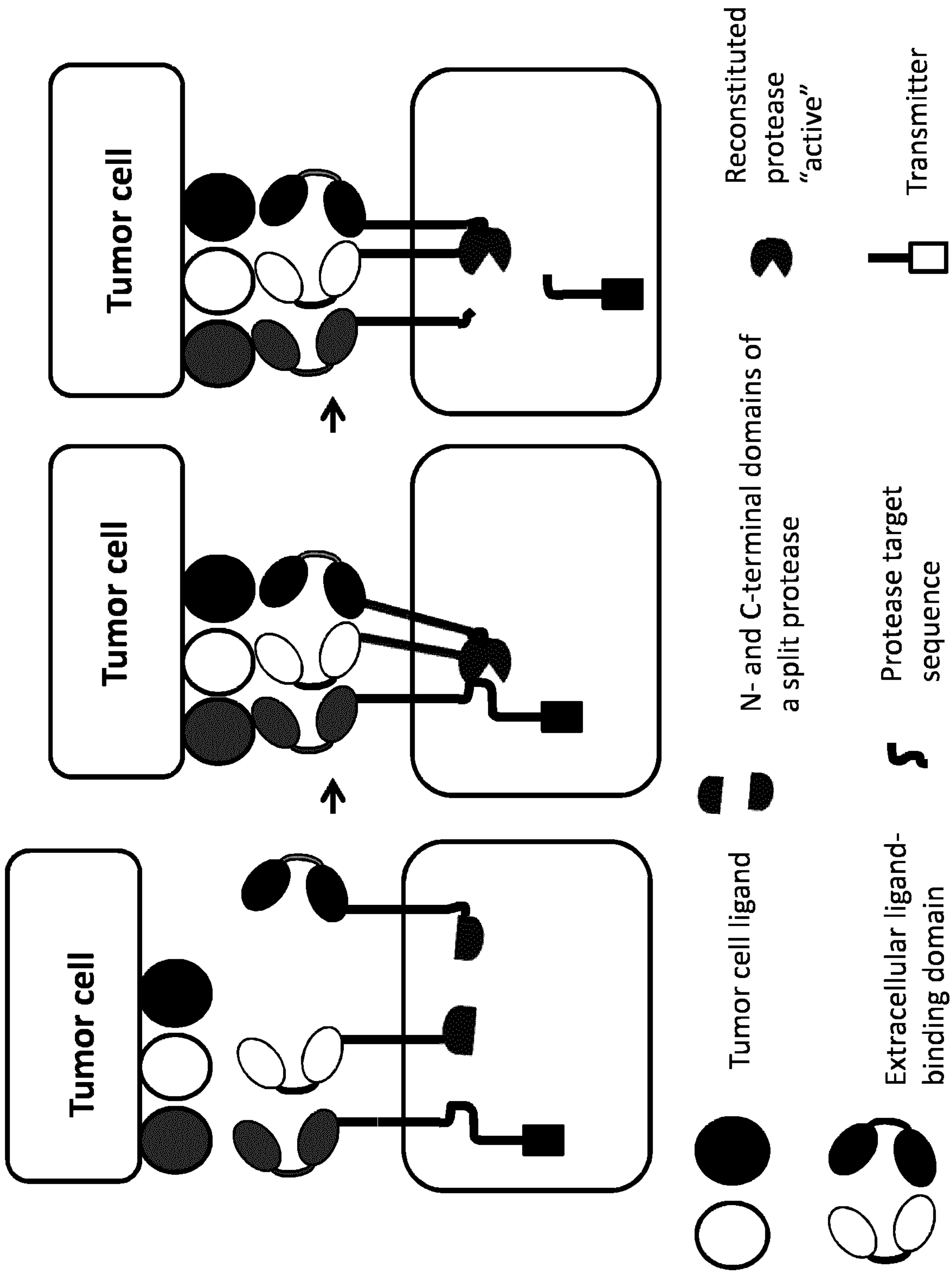


Fig. 10

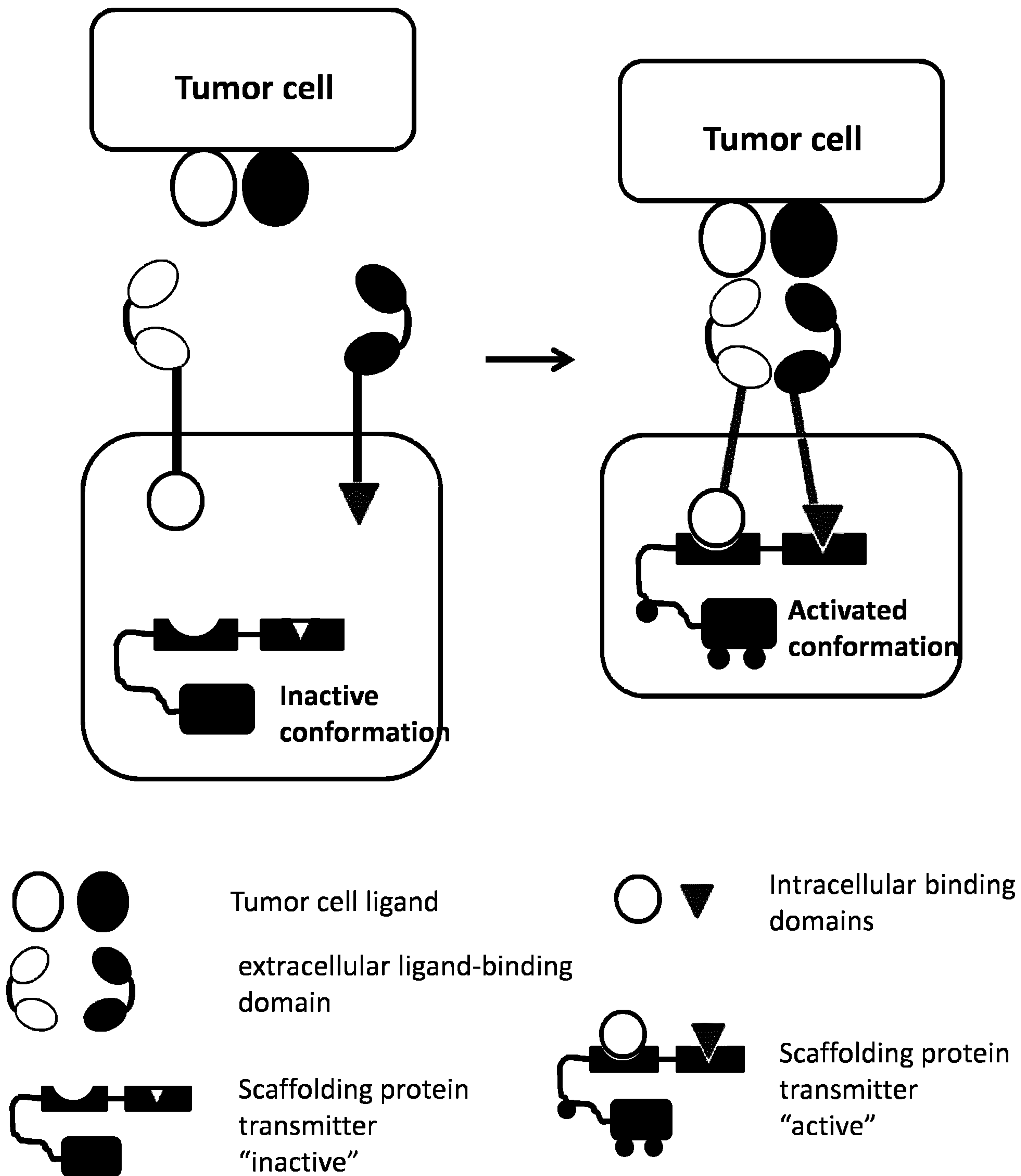
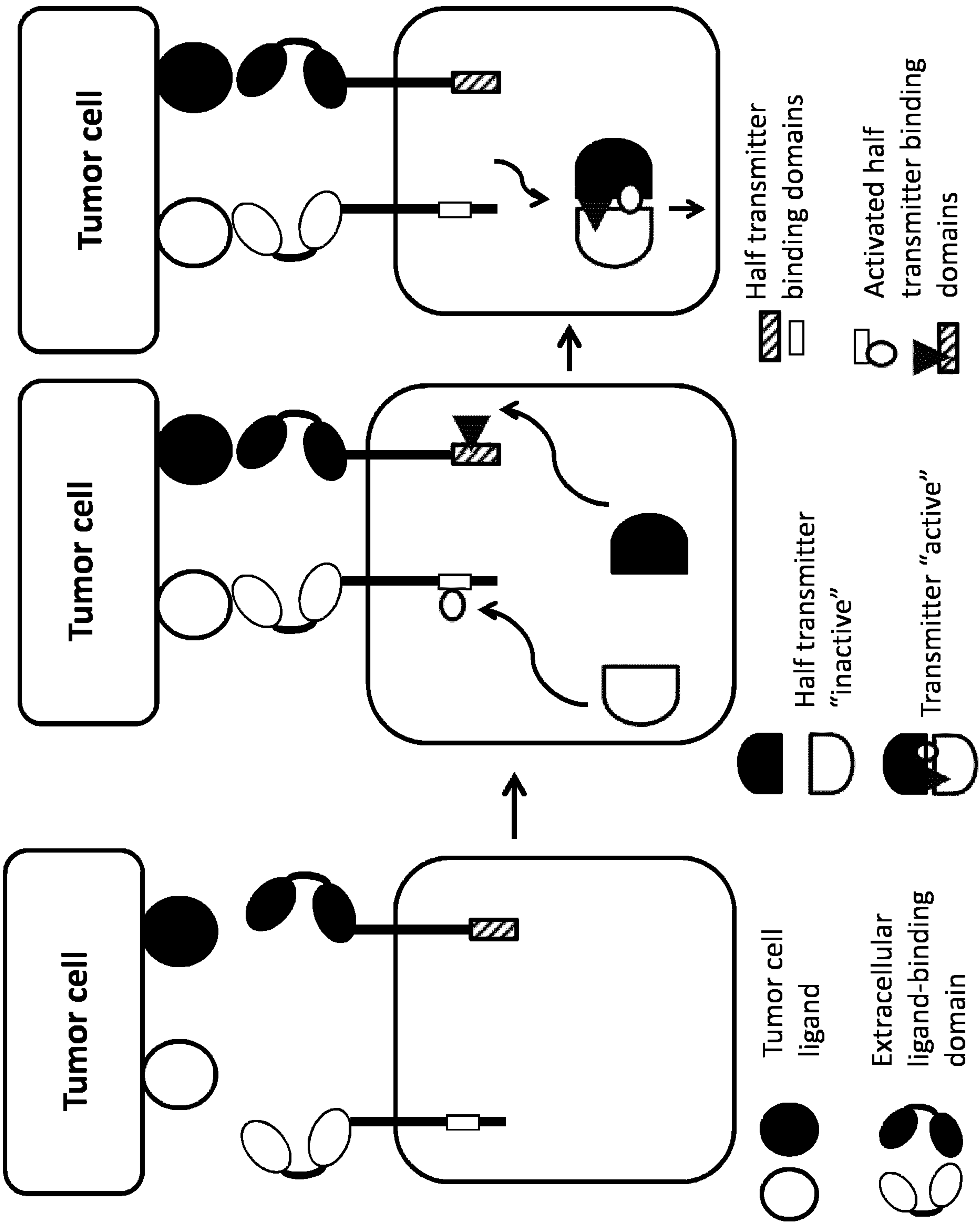


Fig. 11



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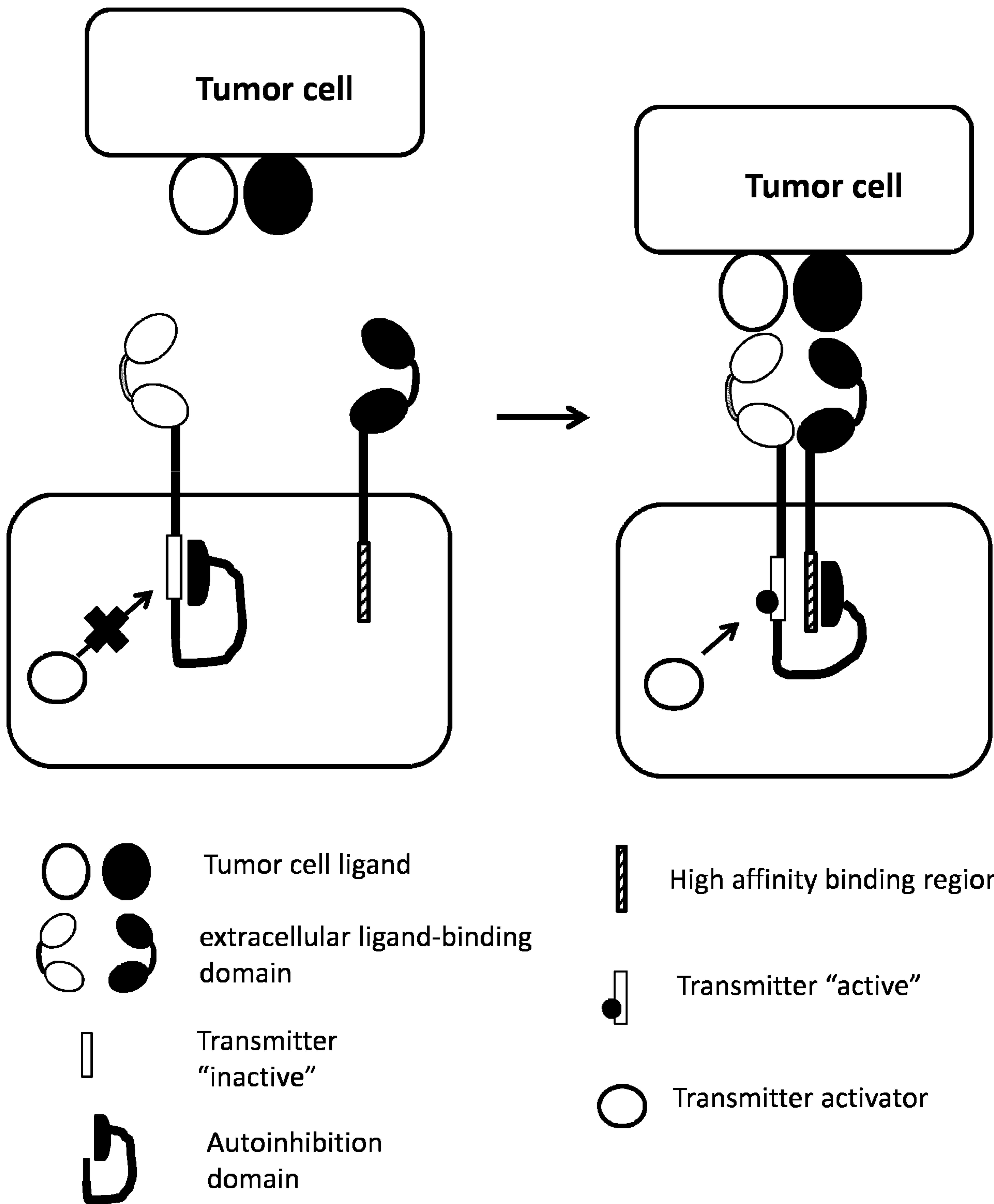


Fig. 13

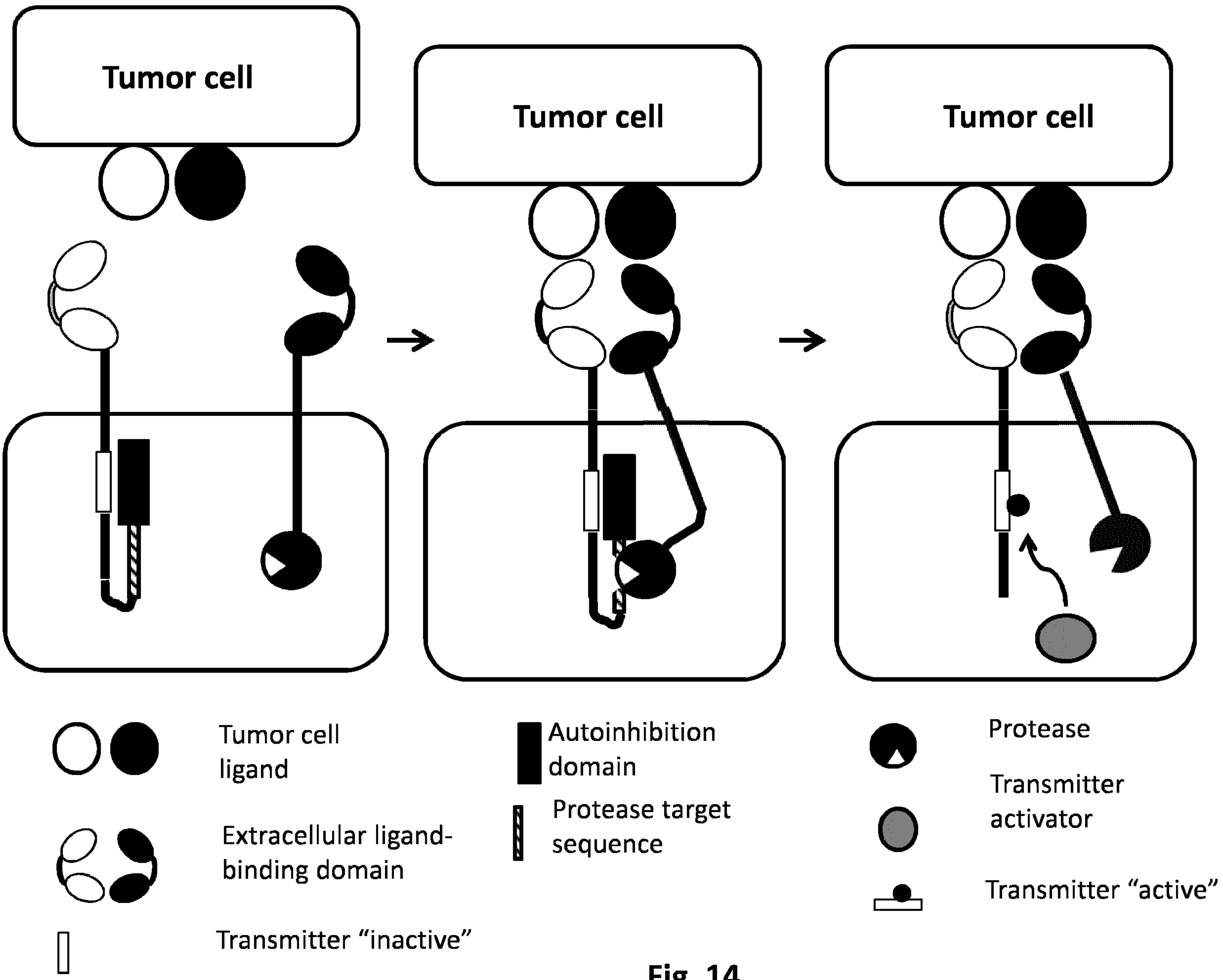


Fig. 14

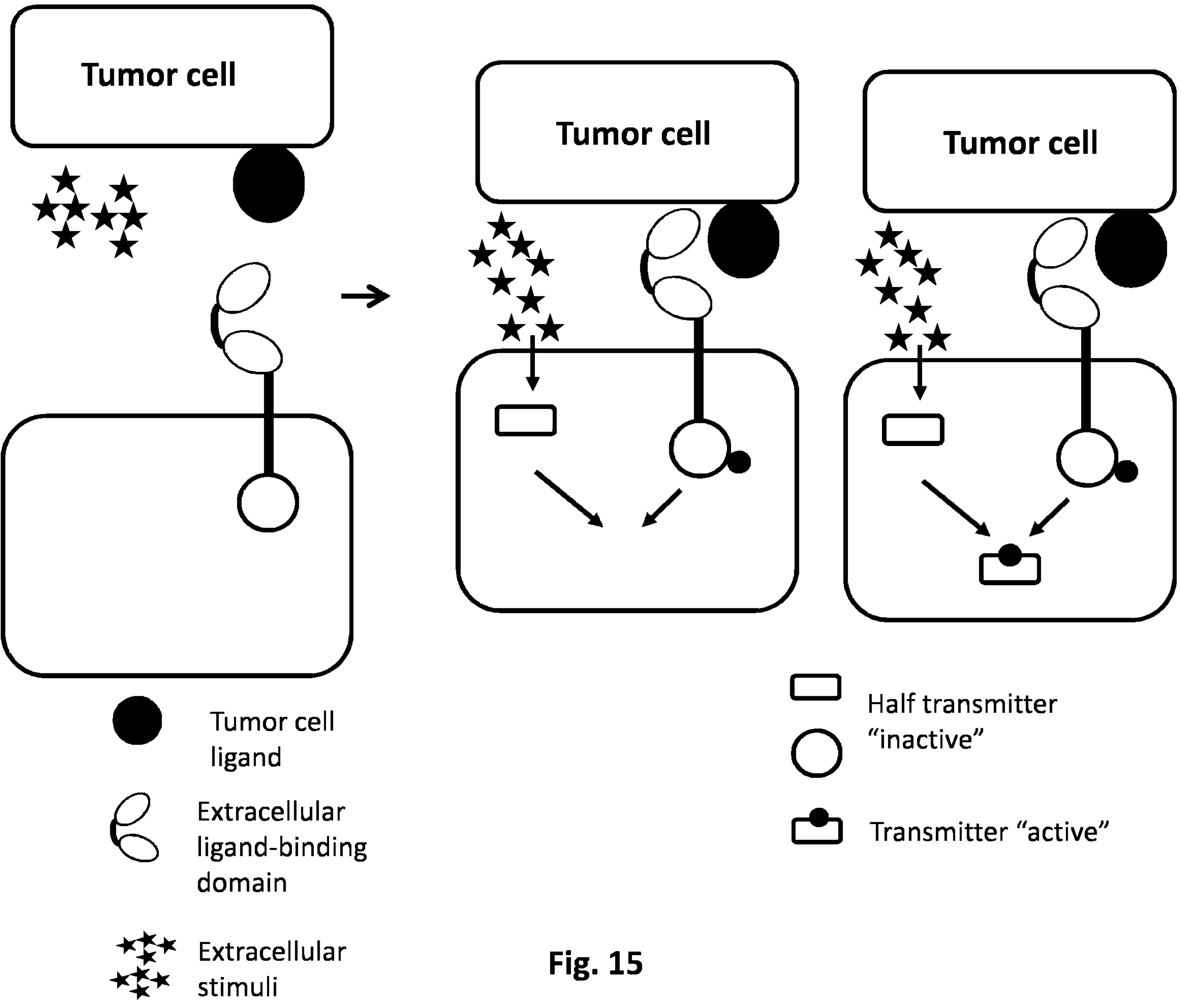


Fig. 15

Hypoxia dependant expression of CARI specific for Antigen 1

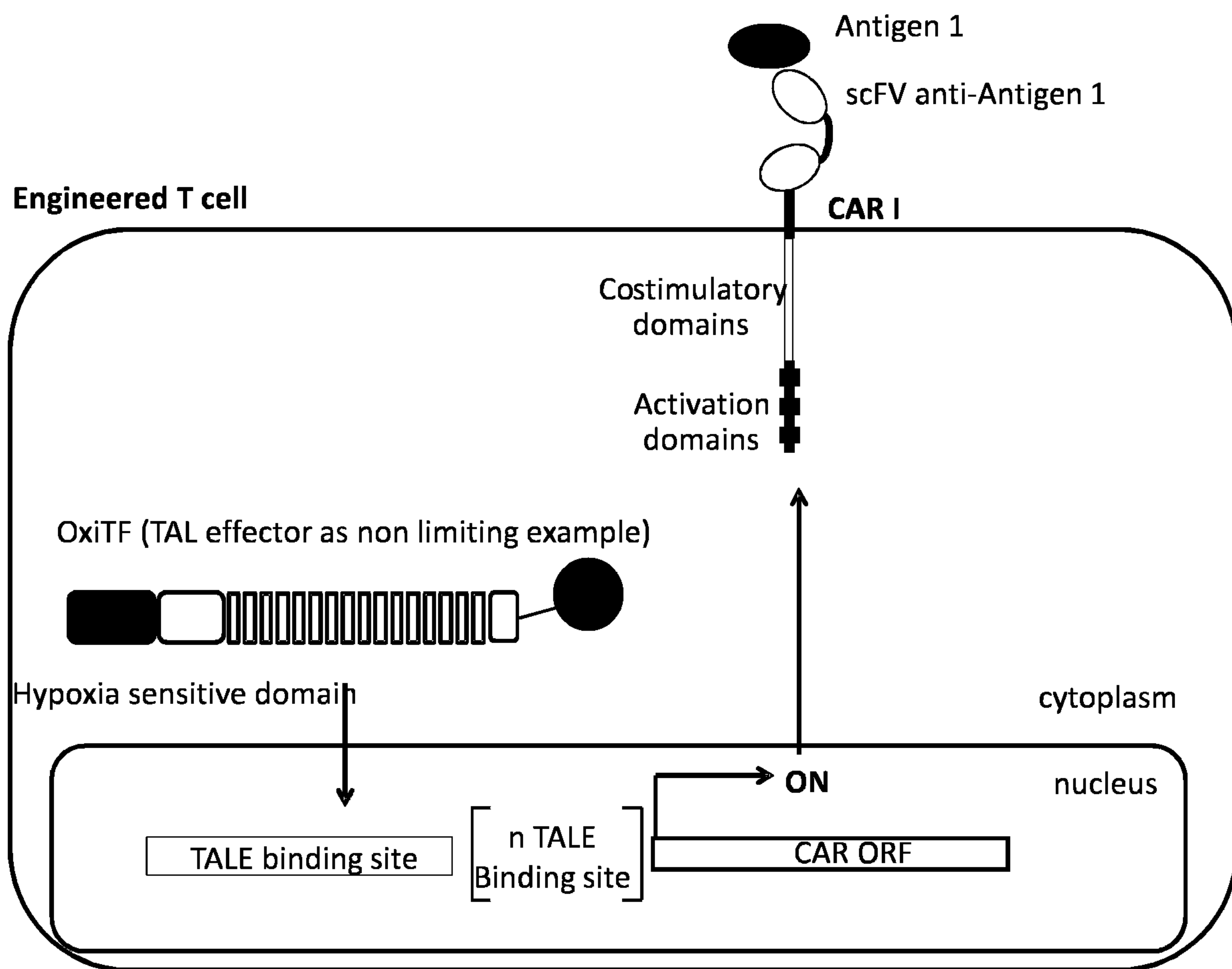


Fig. 16

Constitutive expression of CAR I specific for Antigen 1
AND hypoxia dependent expression of CAR II specific Antigen 2

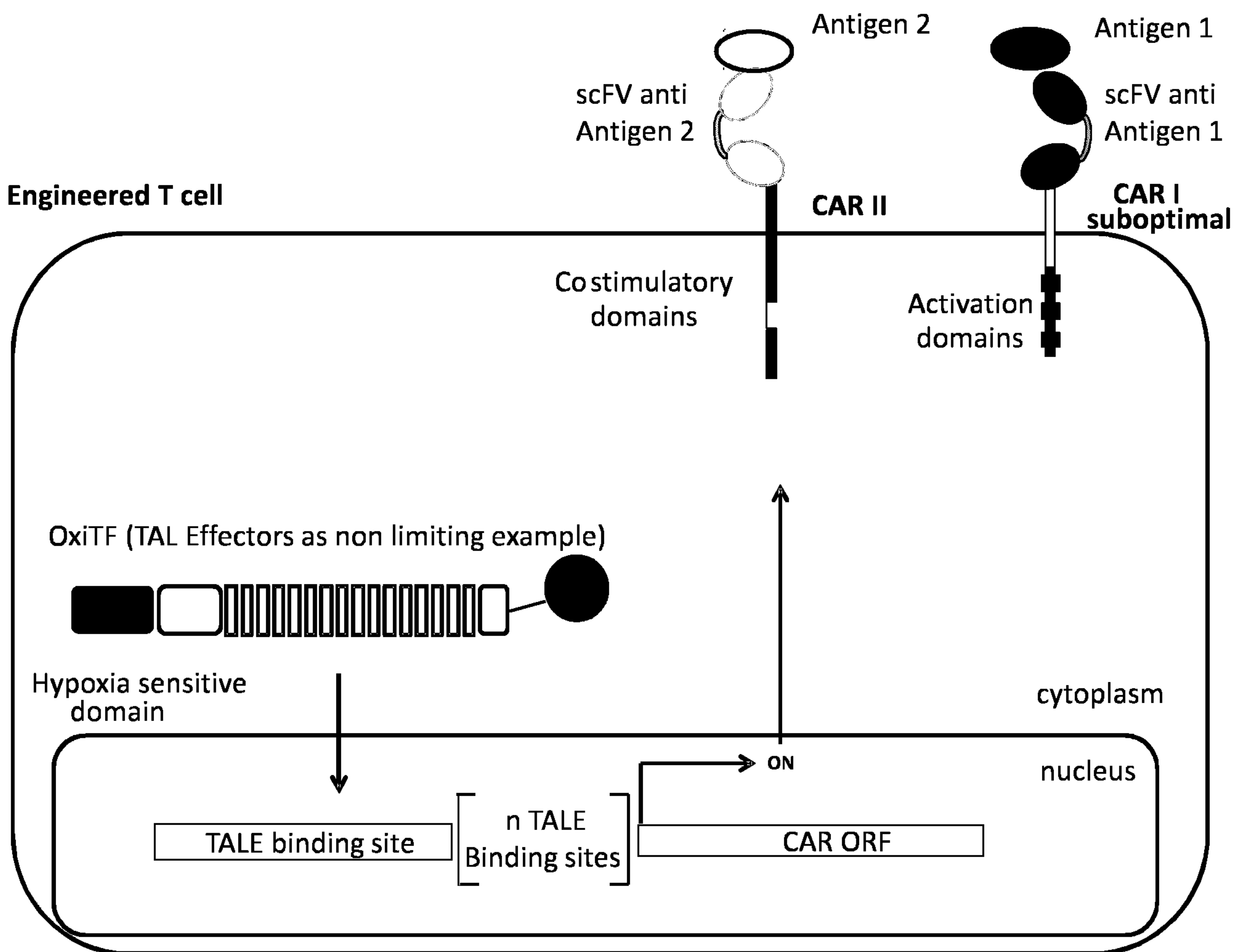


Fig. 17

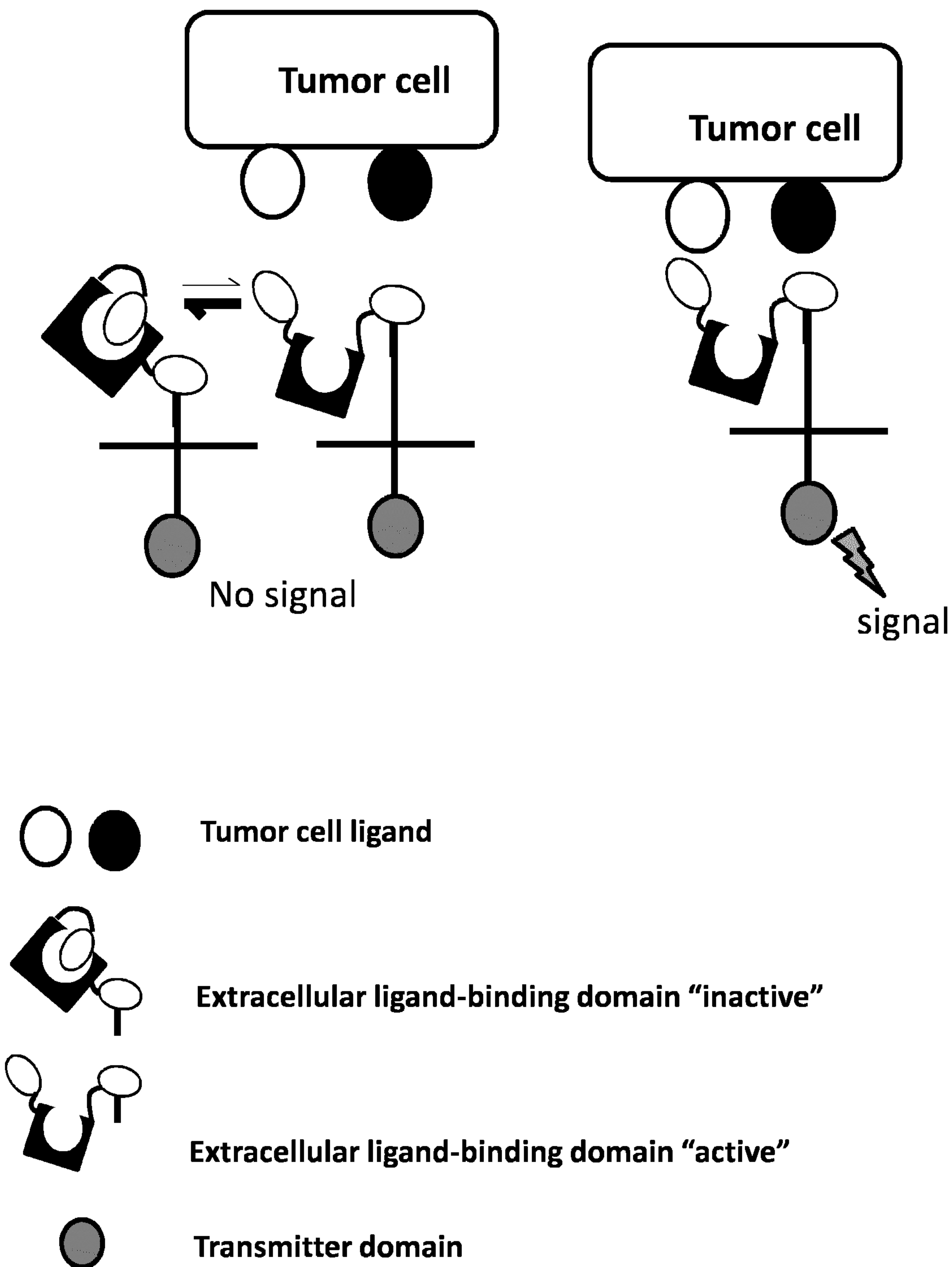
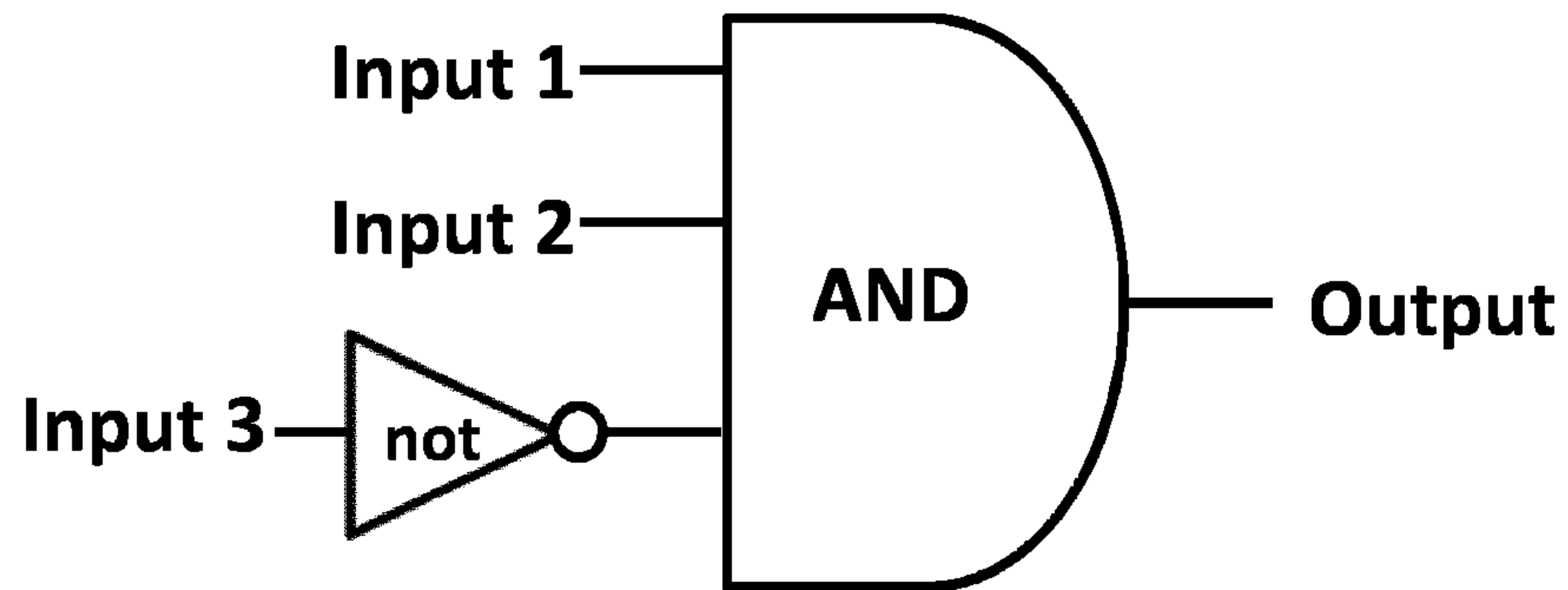


Fig. 18



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0	0	0	0
1	0	0	0
0	1	0	0
0	0	1	0
1	1	0	1
1	0	1	0
0	1	1	0
1	1	1	0

Fig. 19

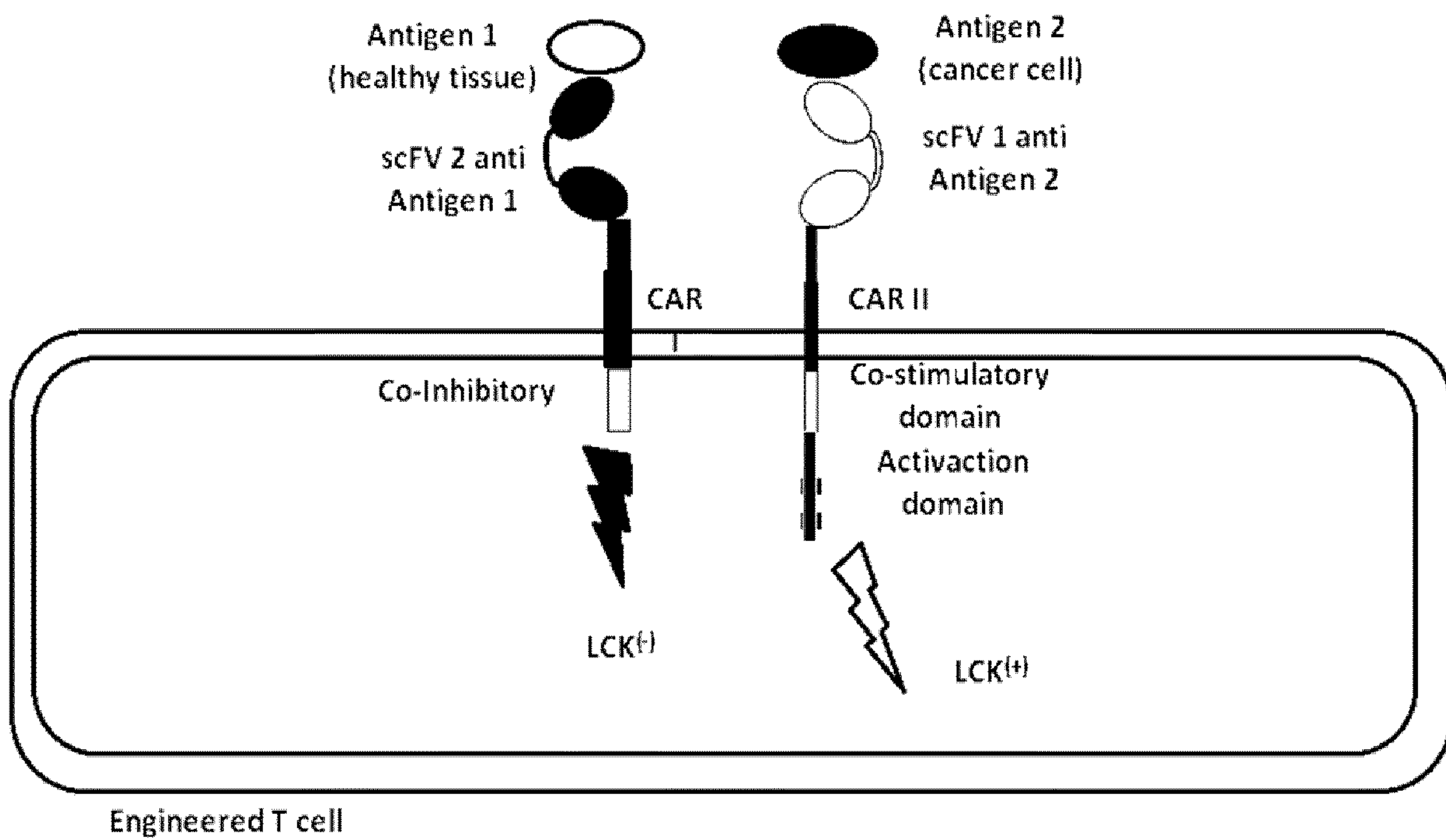


Fig. 20

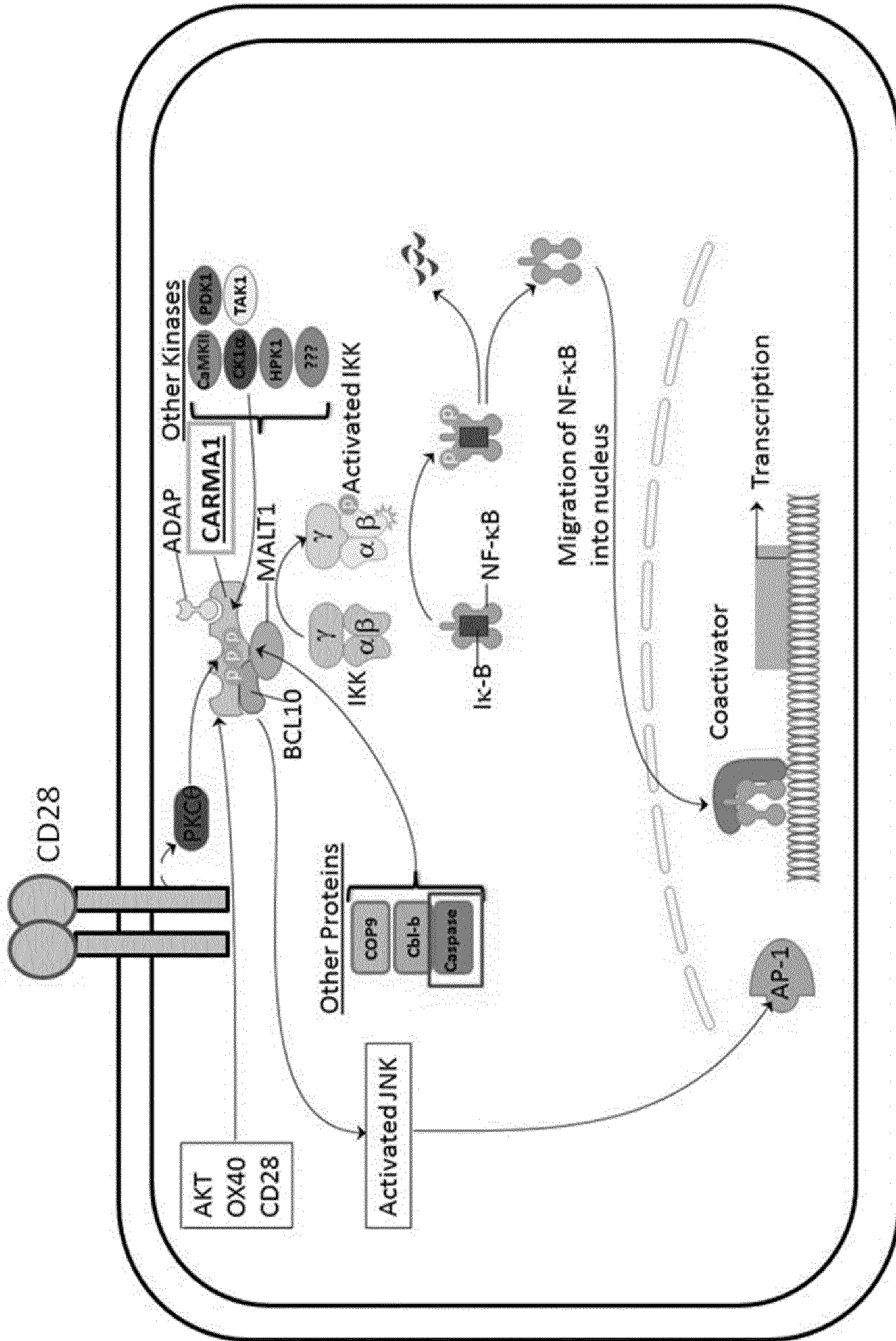


Fig. 21

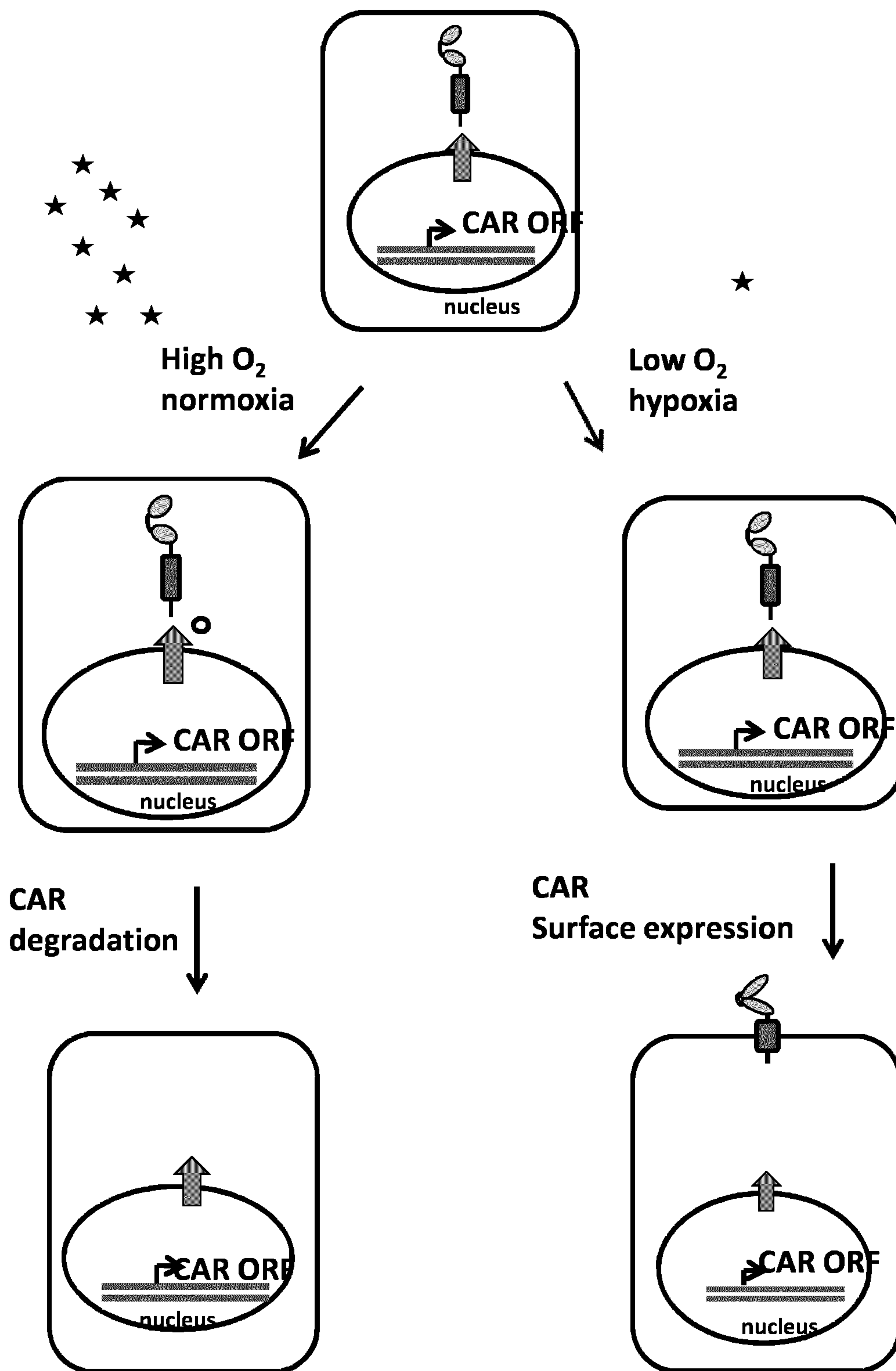


Fig. 22

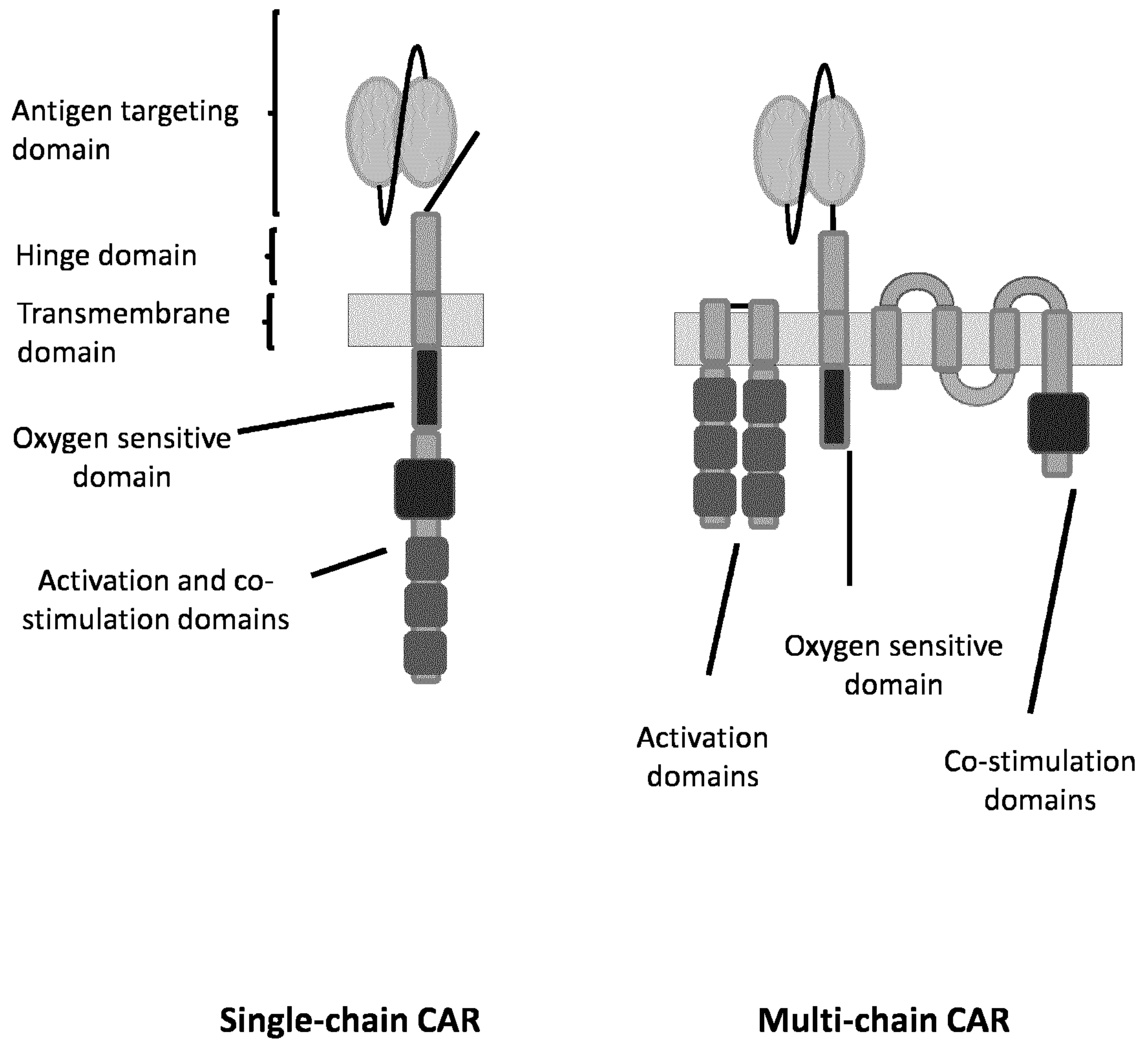
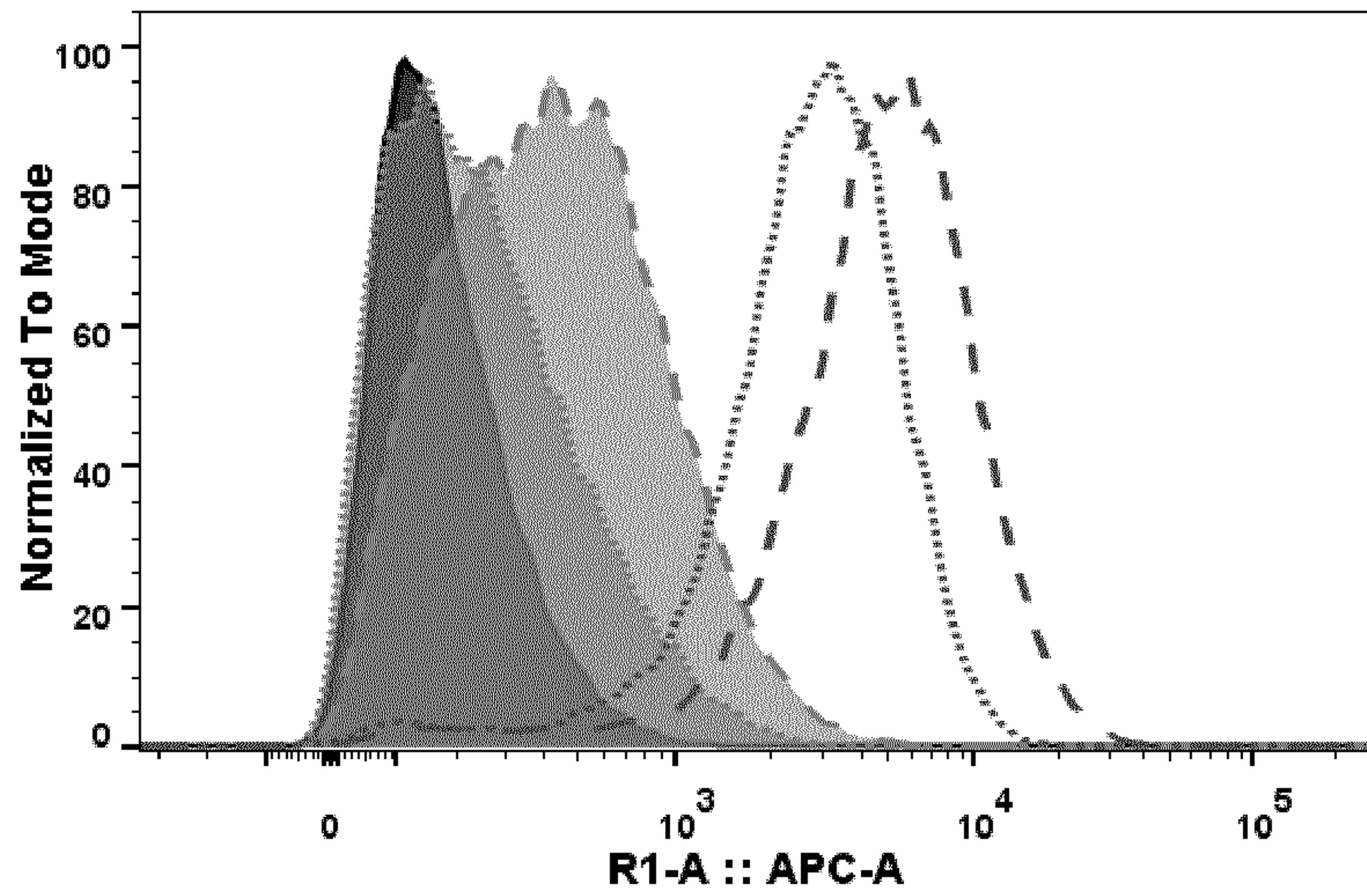


Fig. 23

A



B

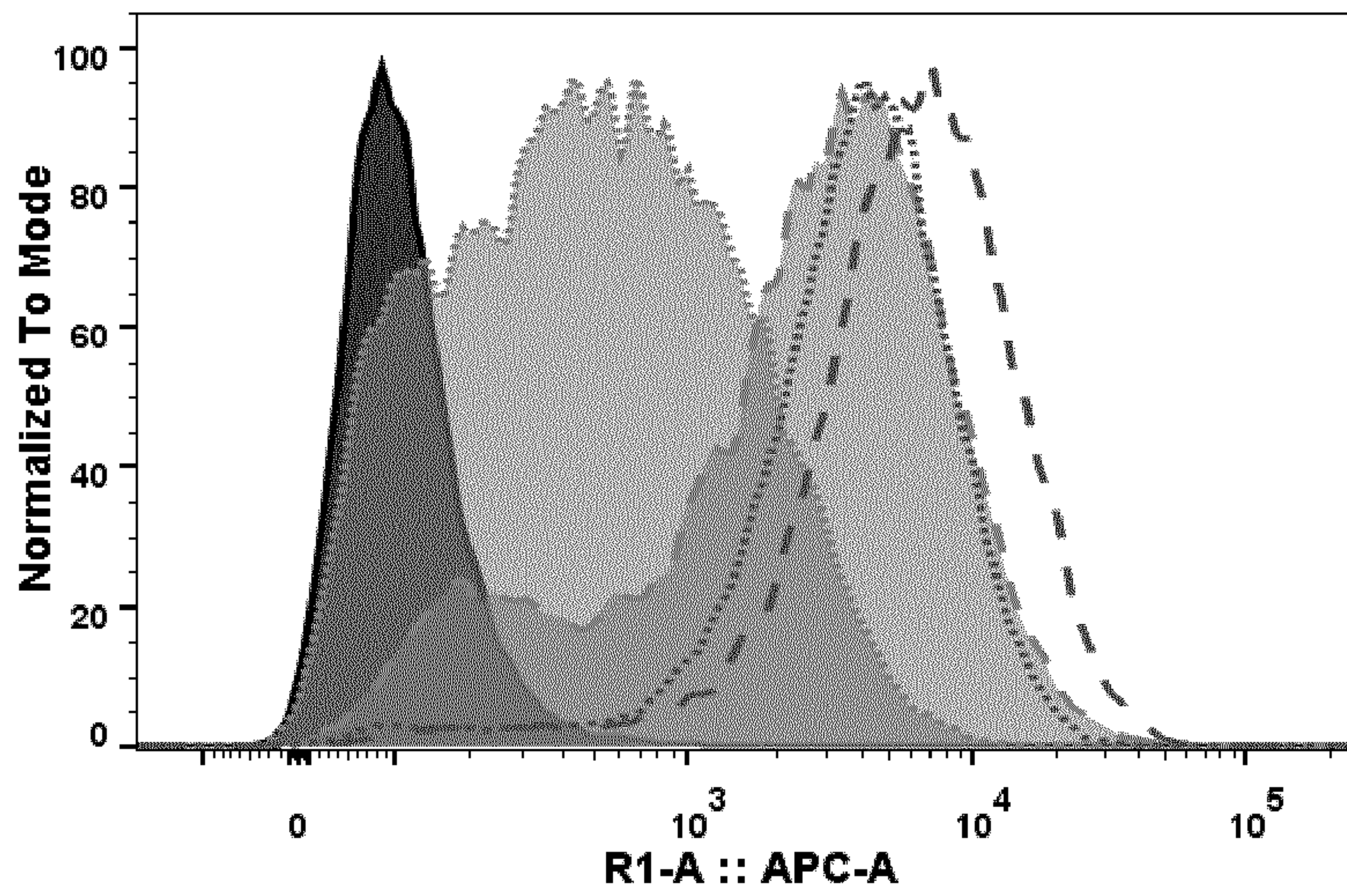
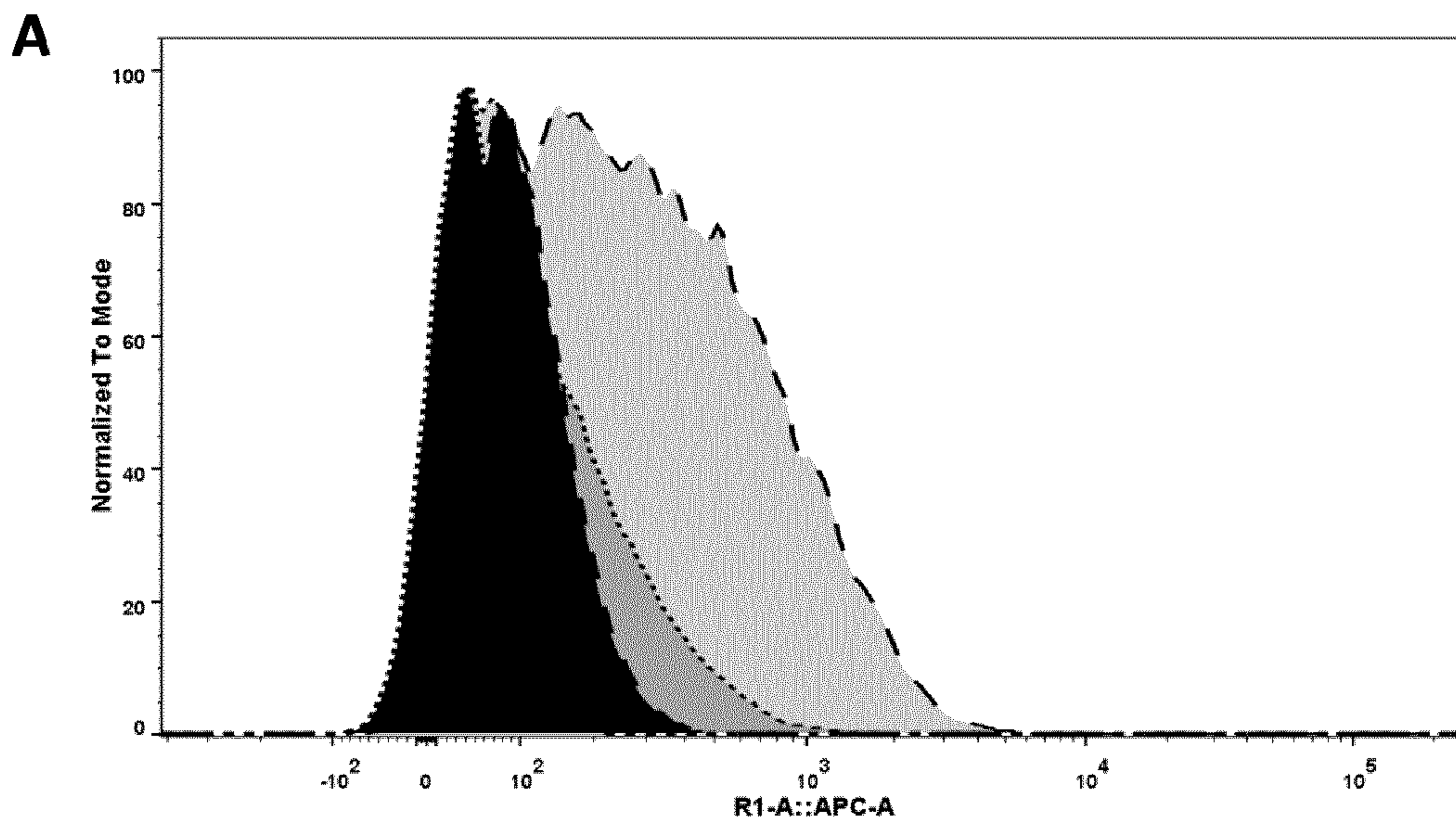


Fig. 24

Sample	Conditions	Histogram
Isotype control	Hypoxia	solide line-dark Filled
α β γ	Normoxia	Dotted line
α β γ	Hypoxia	Dashed line
α-HIF β γ	Normoxia	Dotted line-Filled
α-HIF β γ	Hypoxia	Dashed line-Filled

A



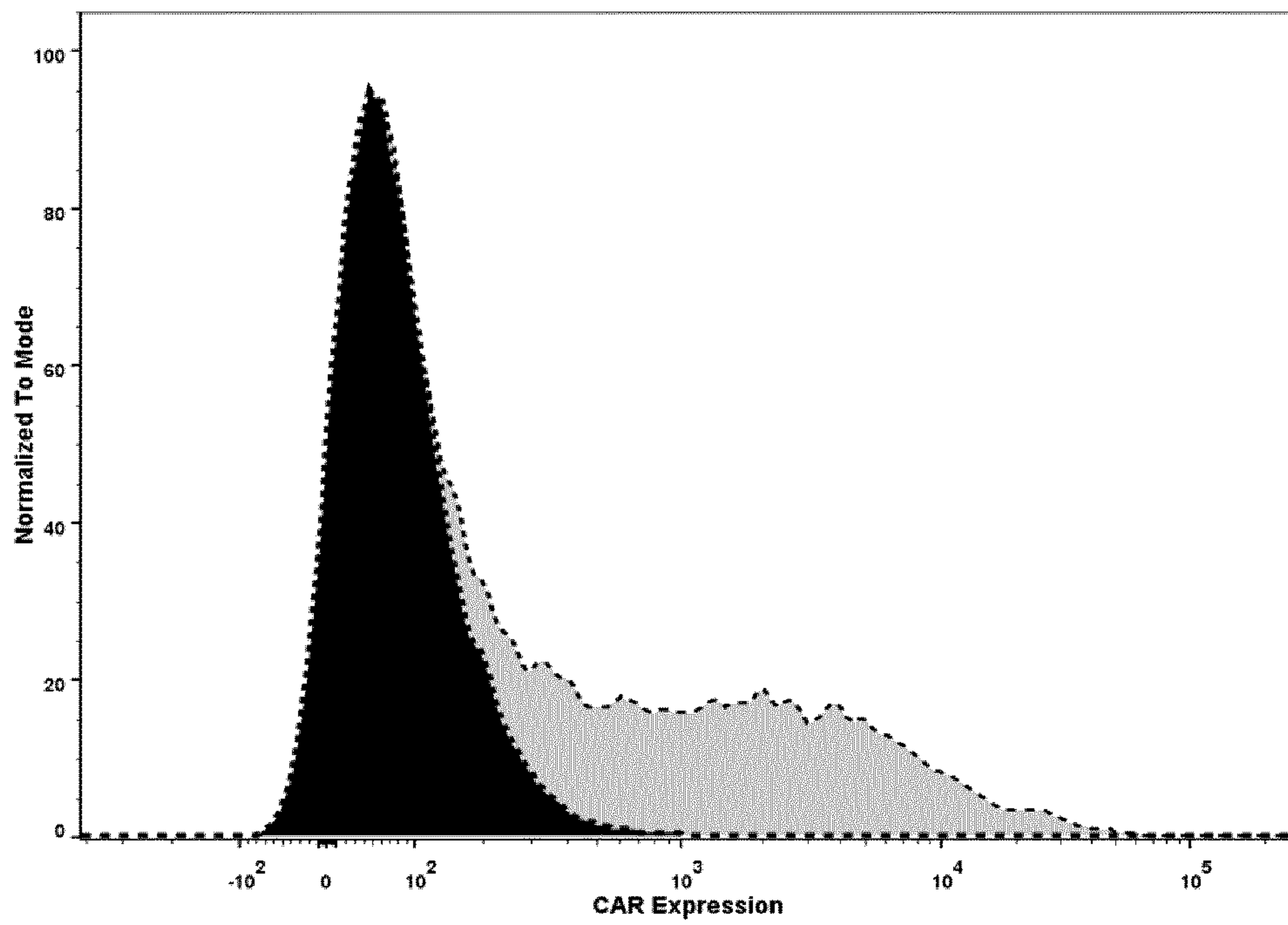


Fig. 25

B

Sample	Target cell killing
HIF-mcCAR	-
Control mcCAR	++

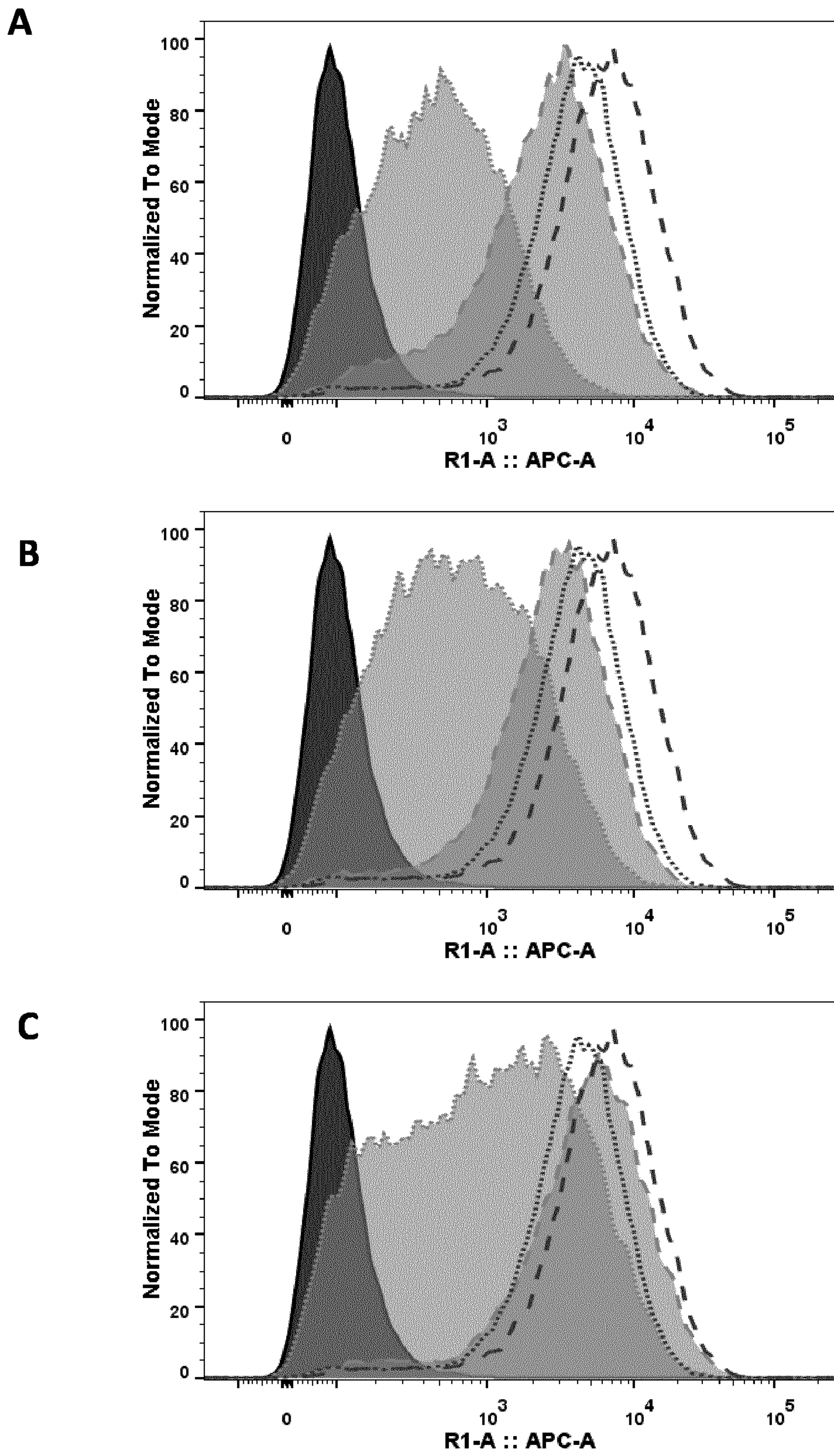


Fig. 26A-C

(The legends are inserted in the figures description)

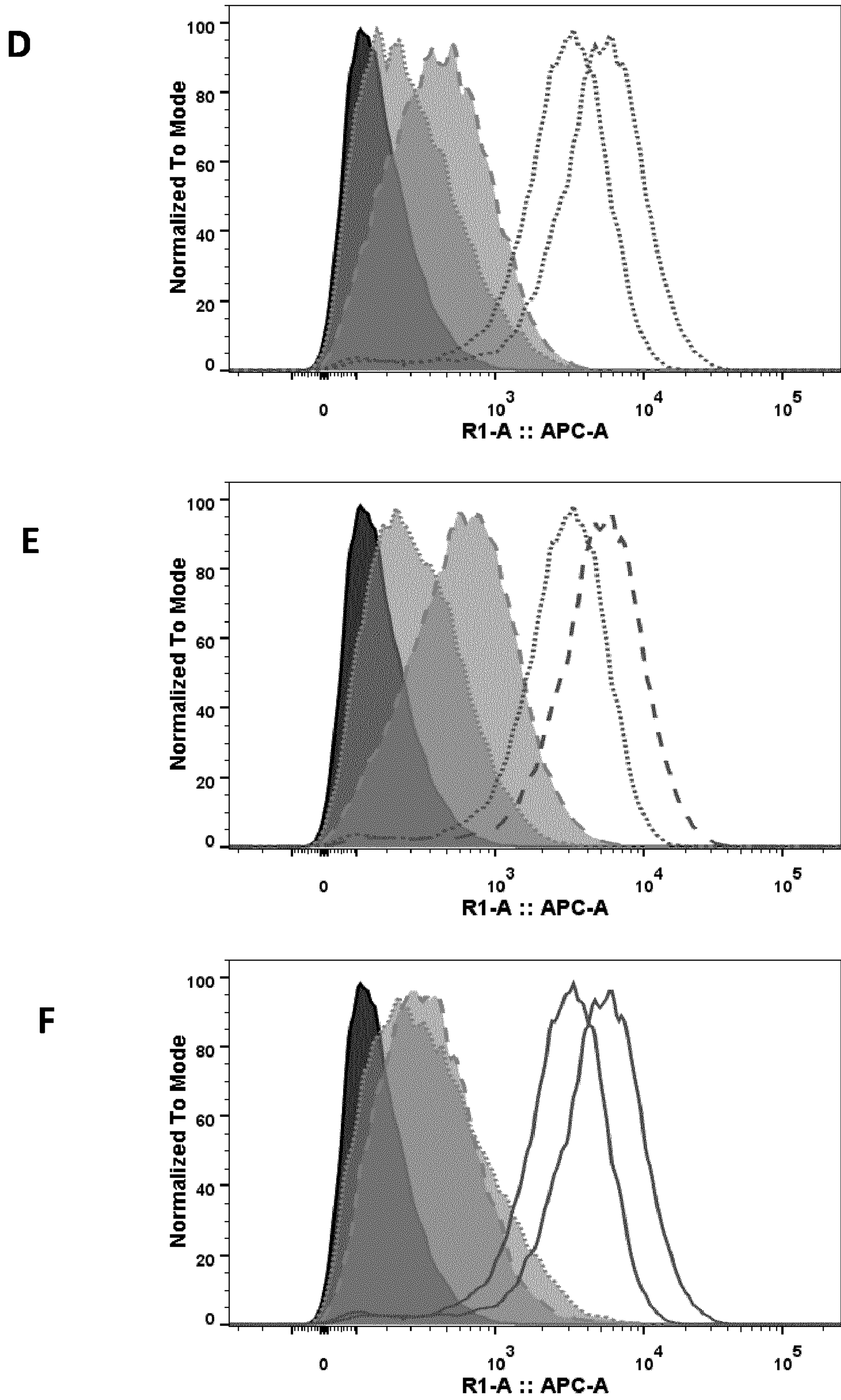


Fig. 26D-F

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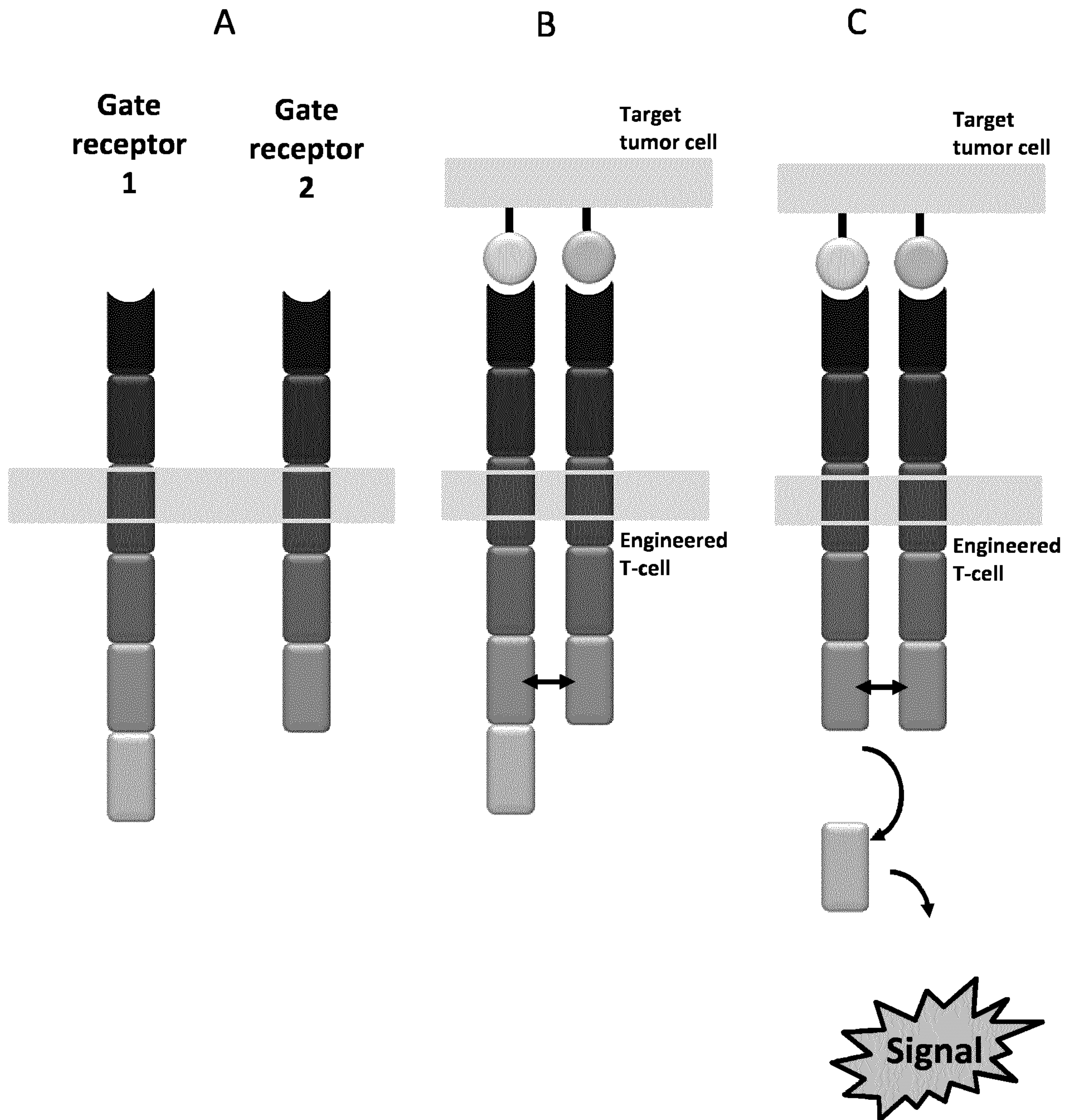


Fig. 27

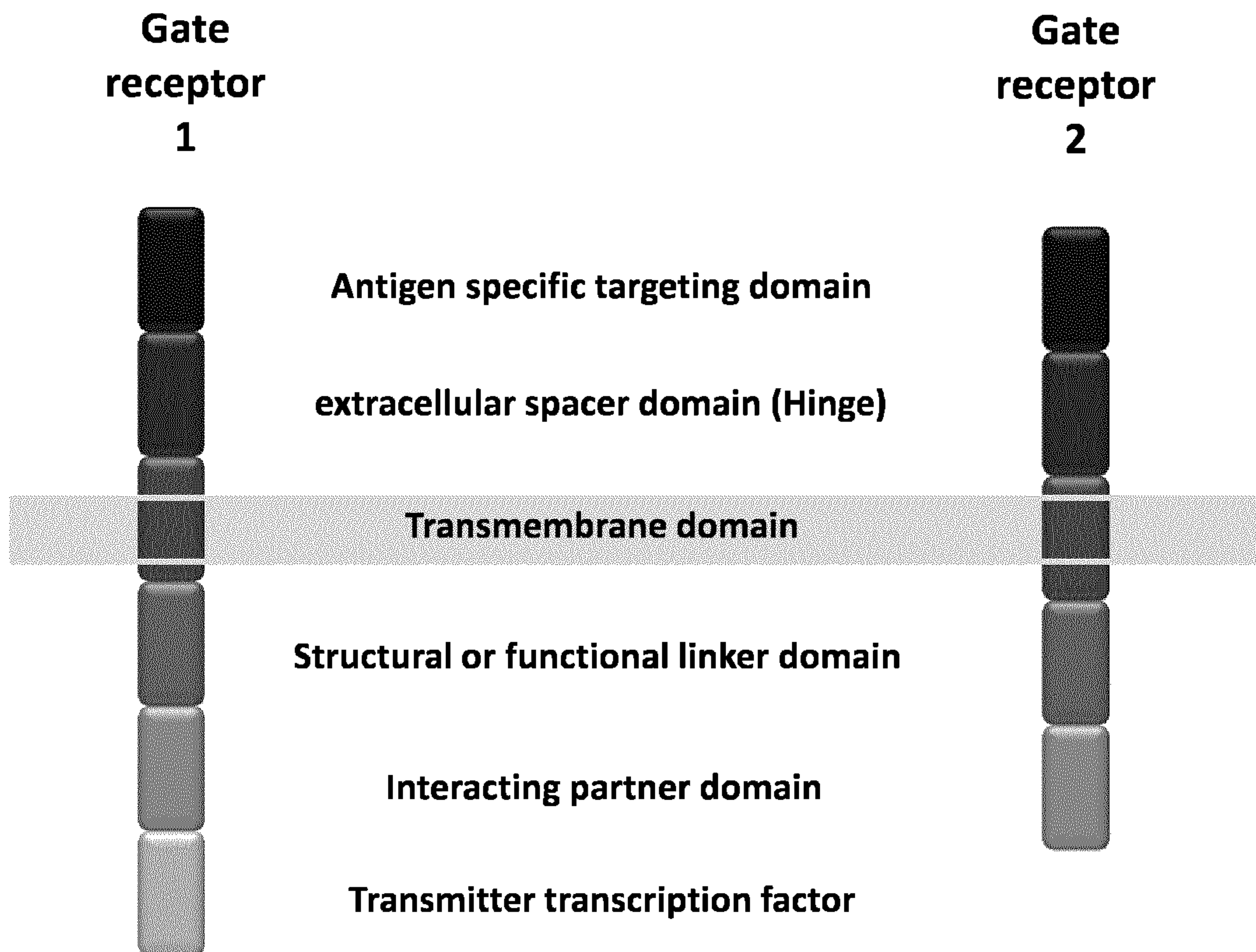


Fig. 28

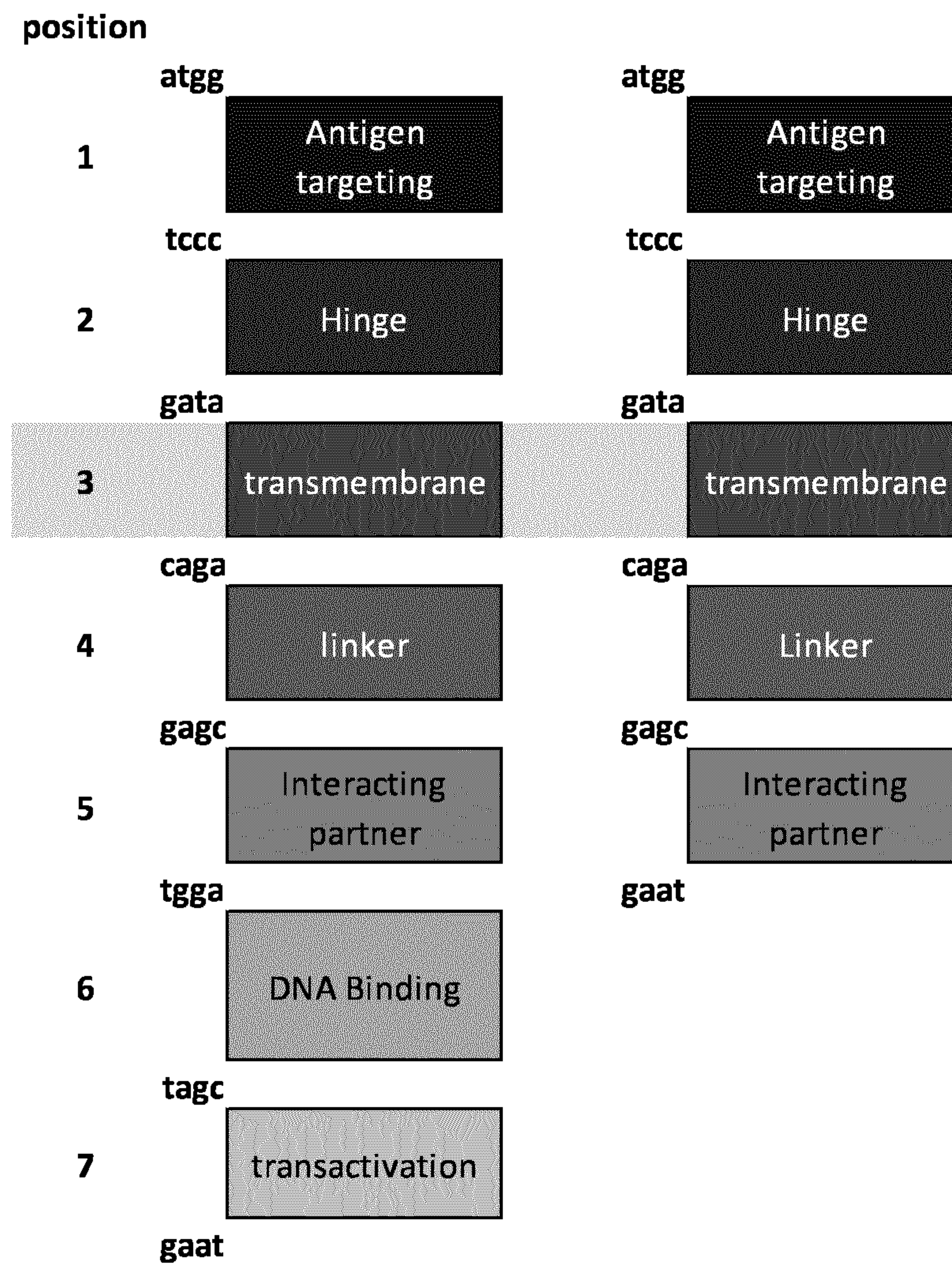


Fig. 29

Membrane protein partner Name	Surface expression
GG83	++
GG111	++
GG121	+
GG152	+
GG153	++
GG155	++
GG156	+
GG158	+

Fig. 30

		Reporter	
		Gal4	TetO
transactivator	Gal4_NF-Kb	+	-
	GAL4_VP64	++	-
	TetR_NF-Kb	-	+
	TetR_VP64	-	++

Fig. 31

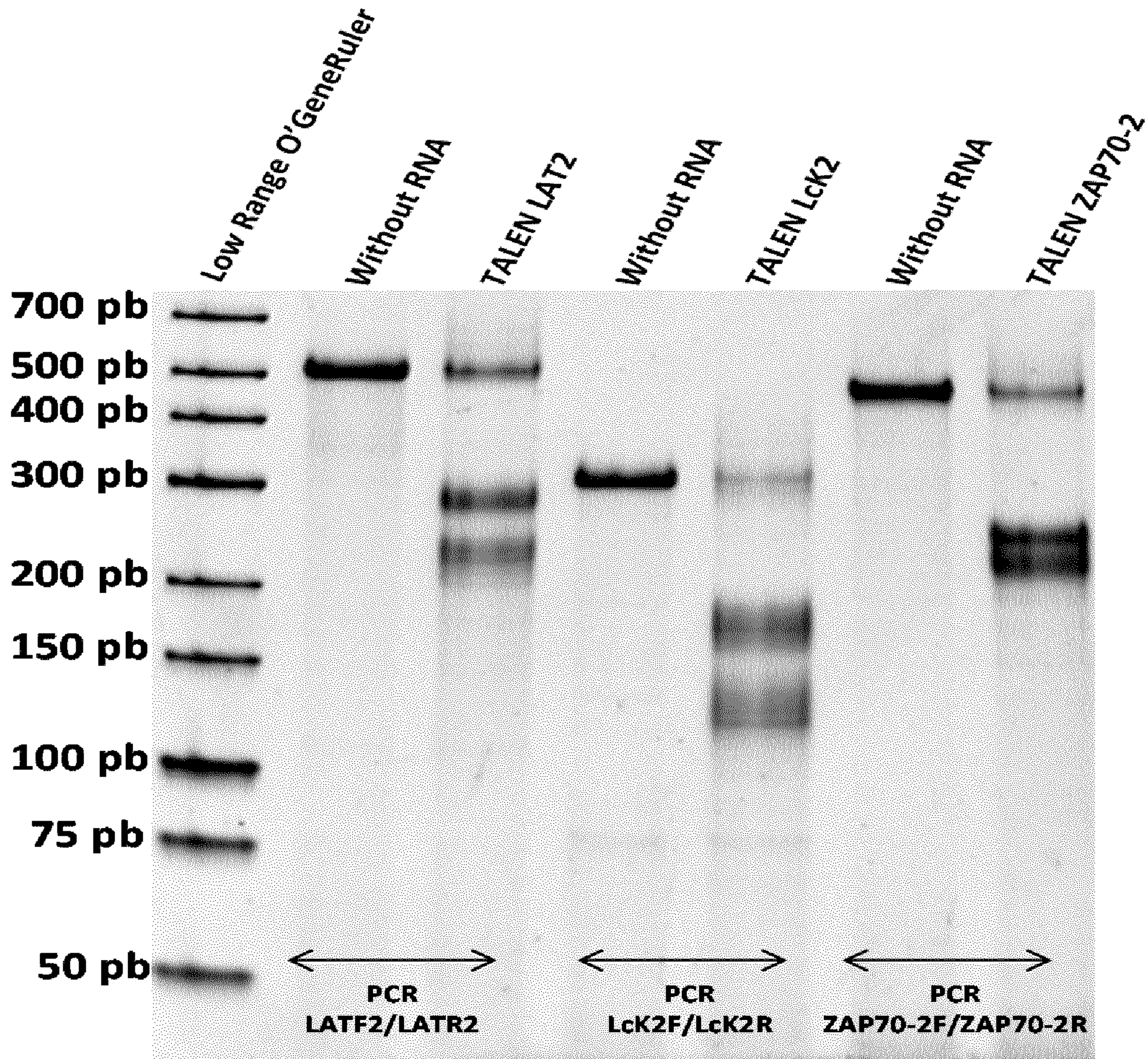


Fig. 32A

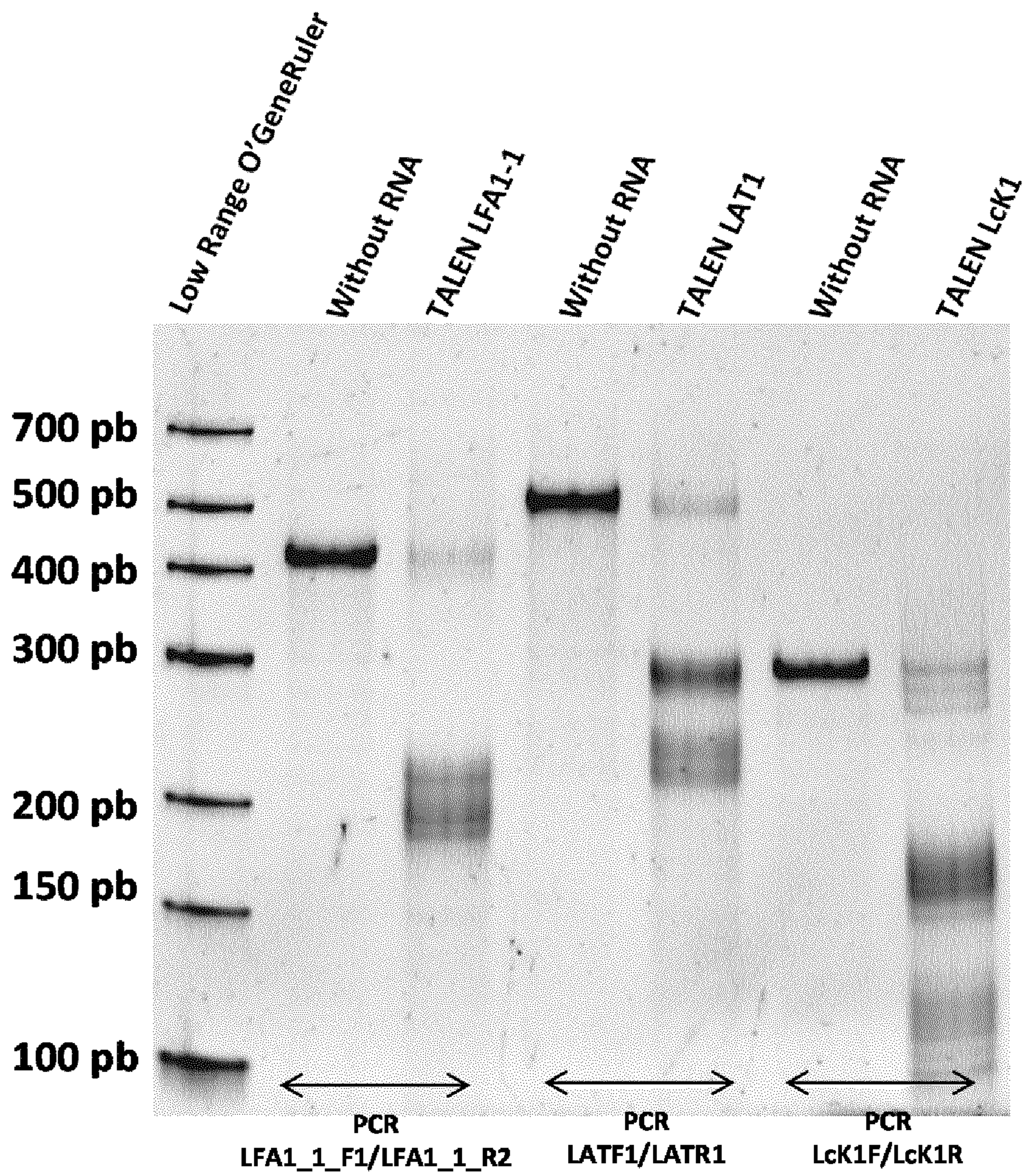


Fig. 32B

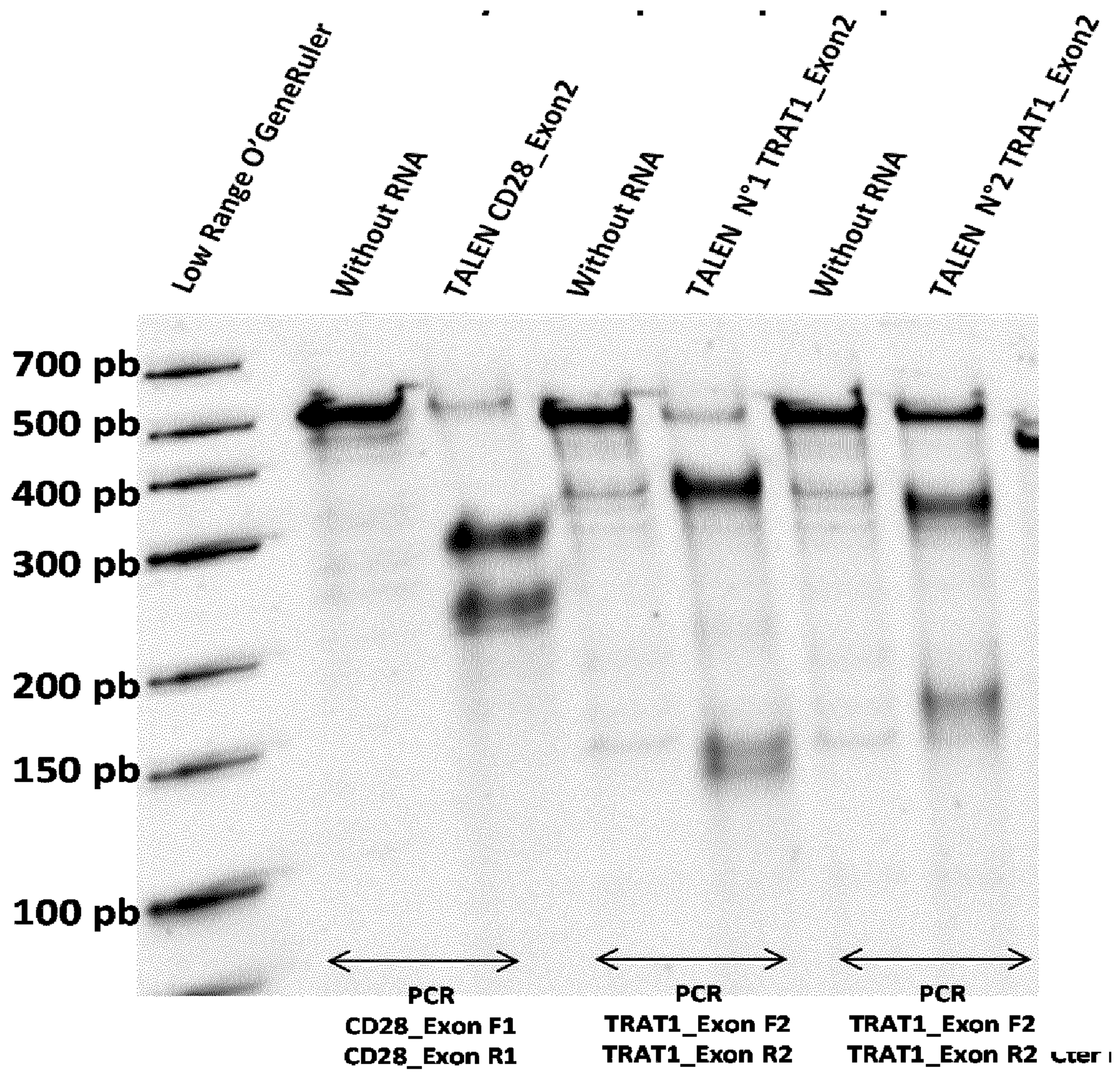
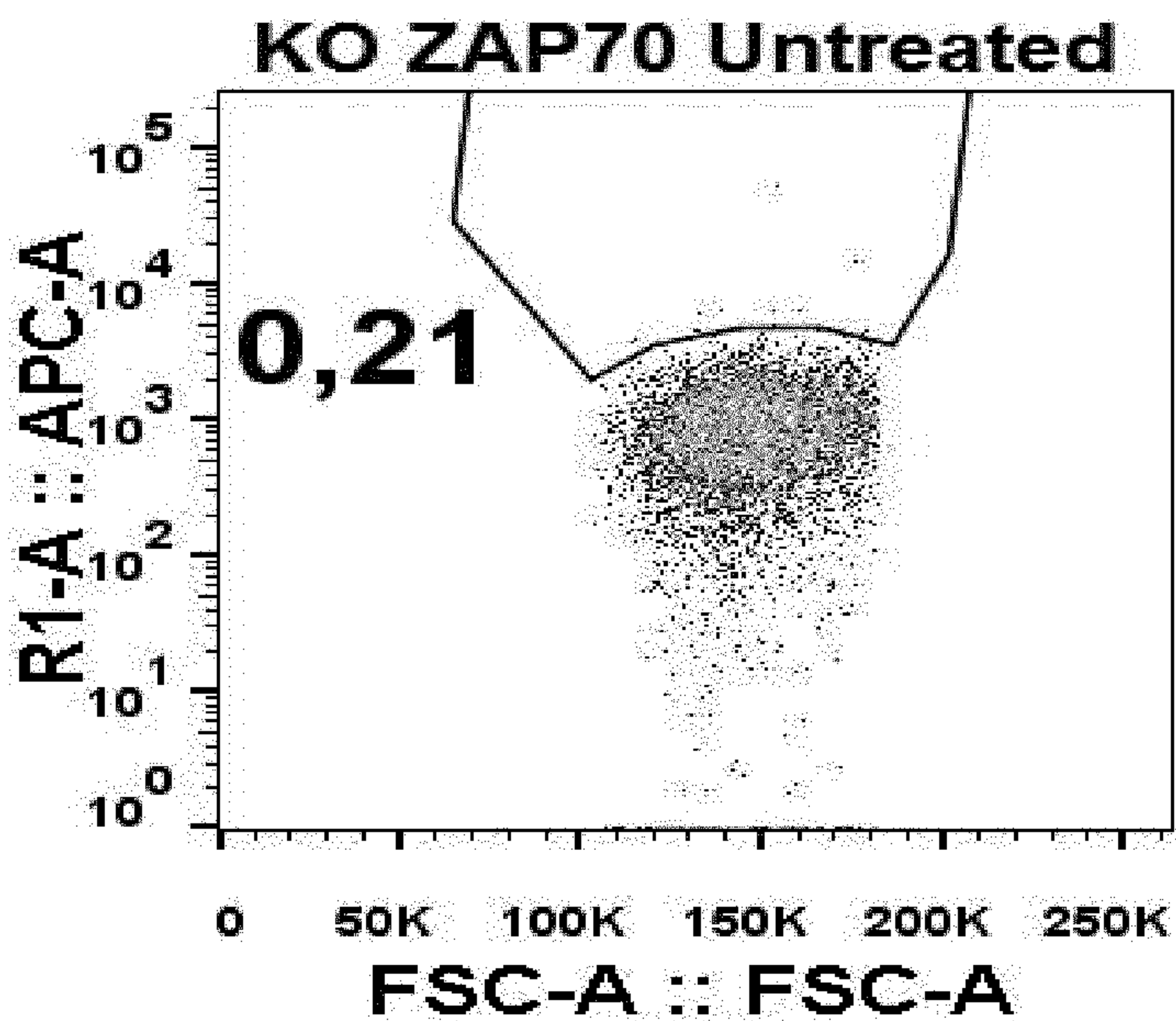
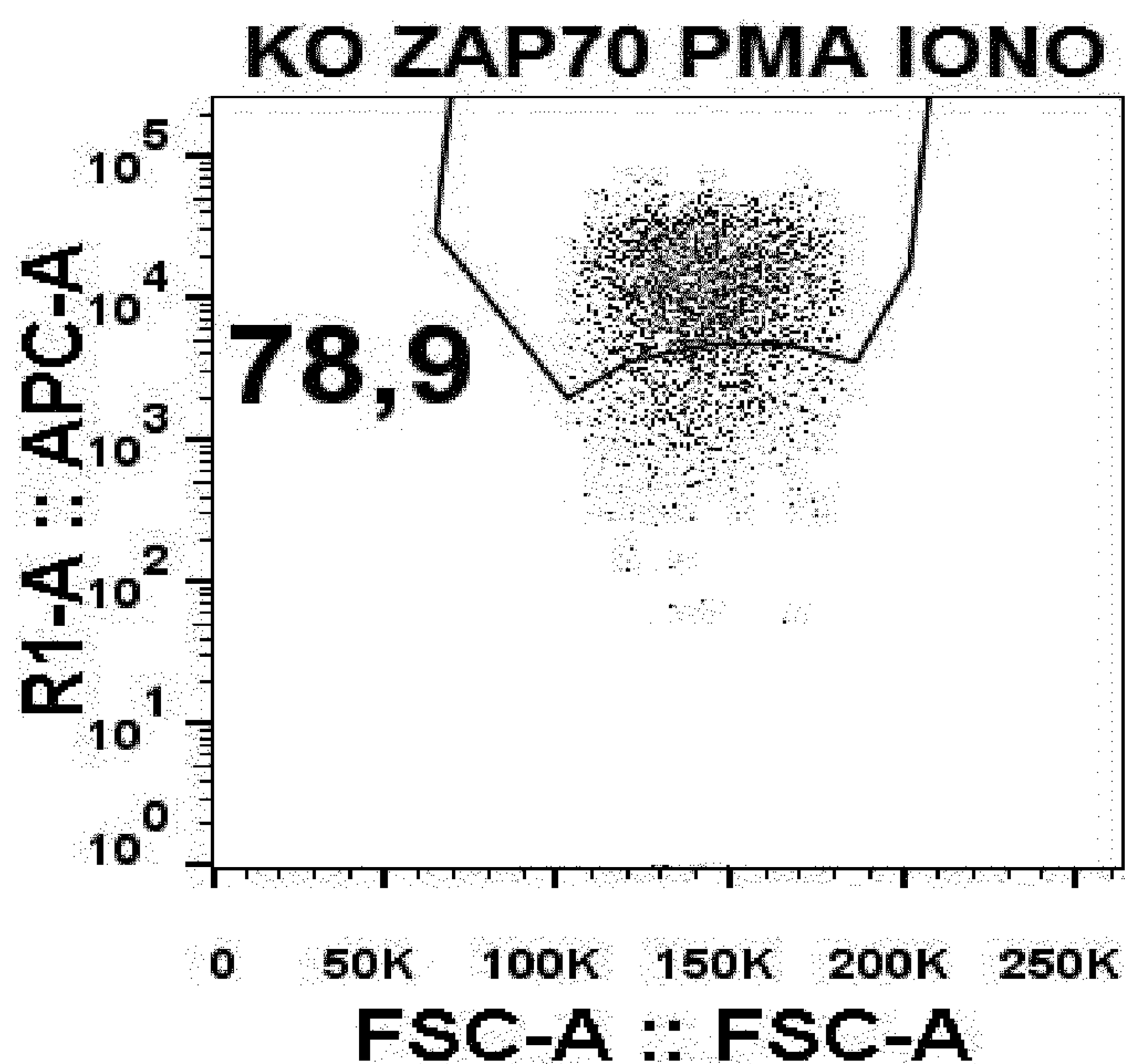
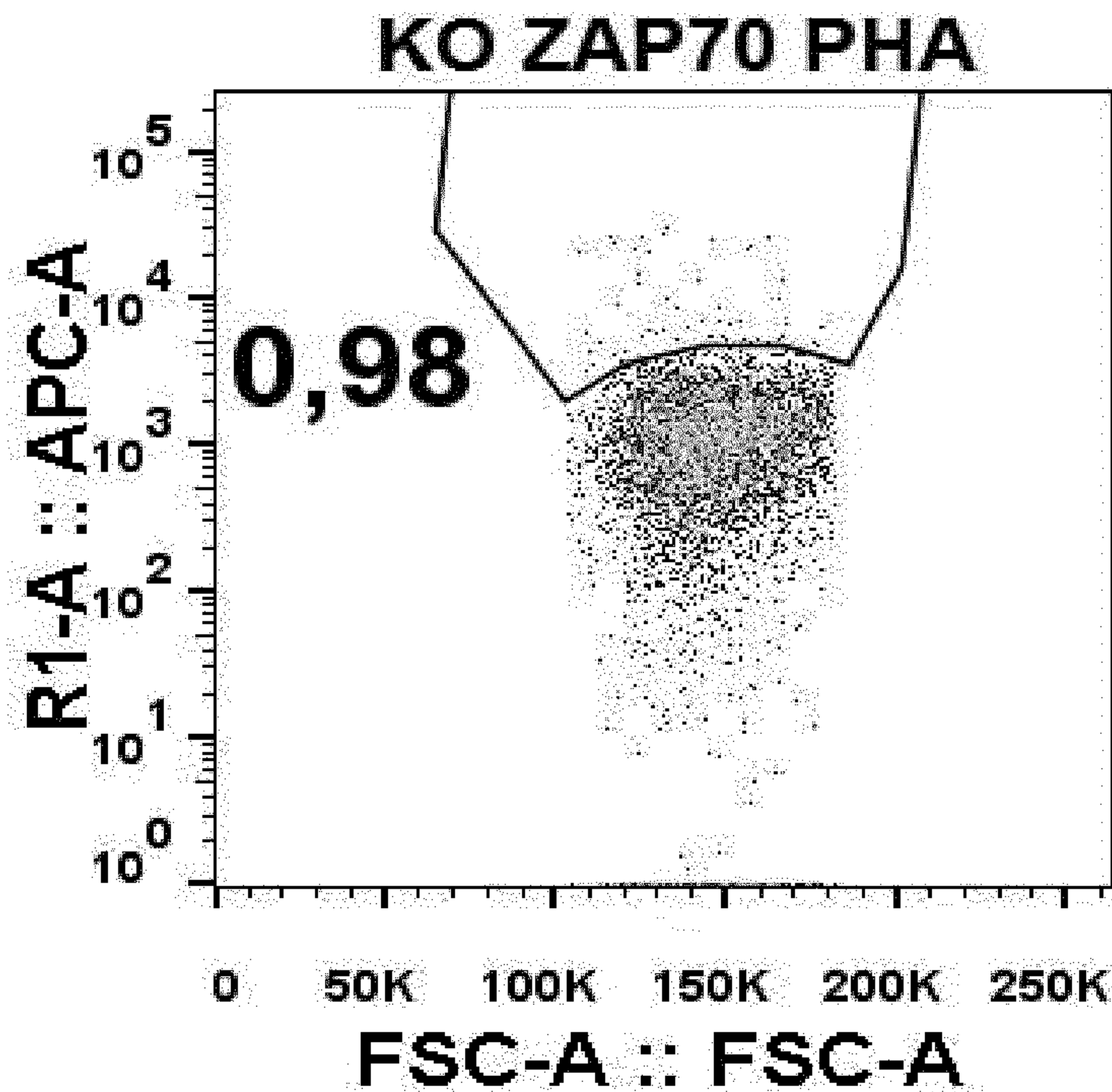


Fig. 32C

KO ZAP70







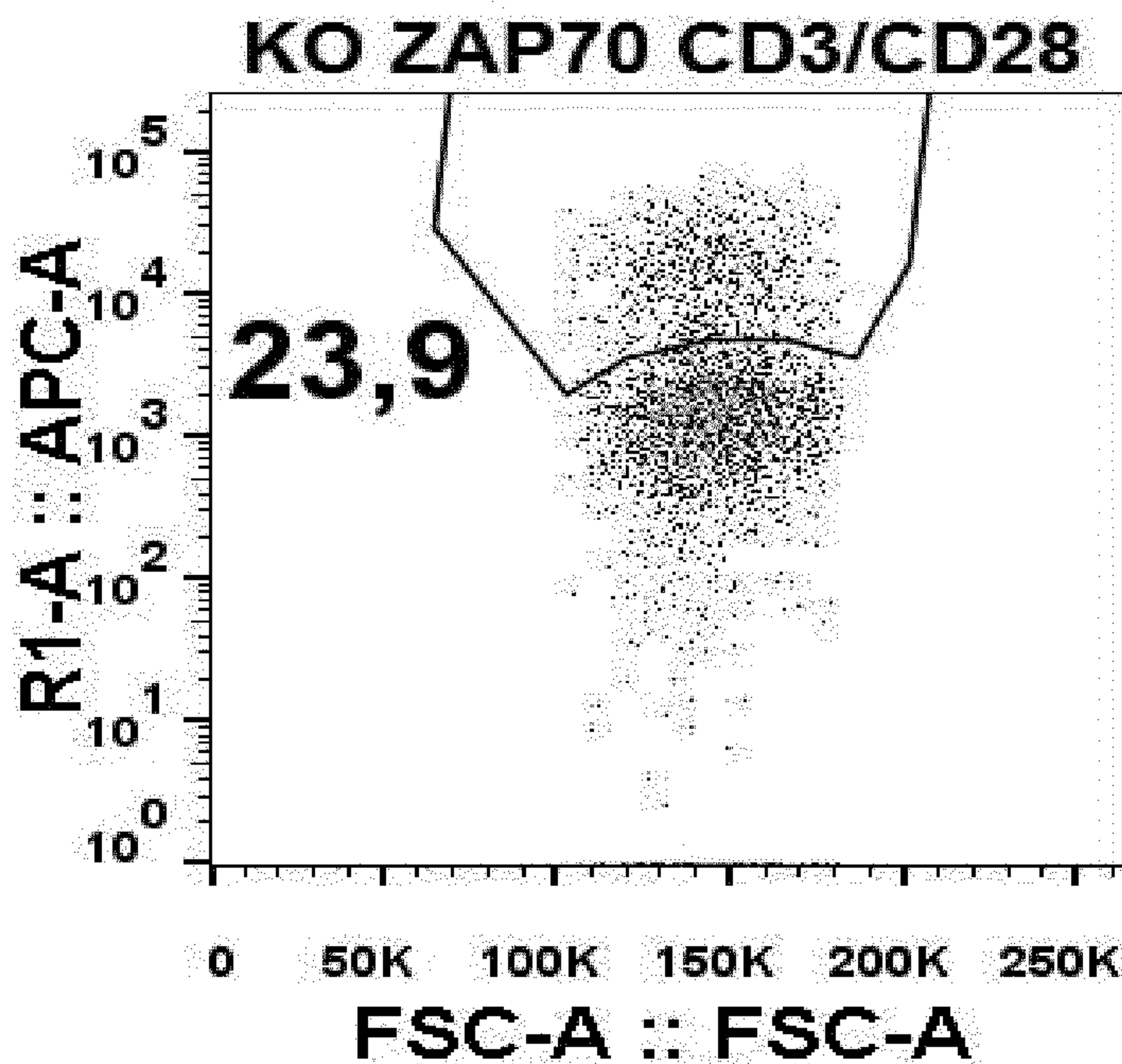


Figure 33

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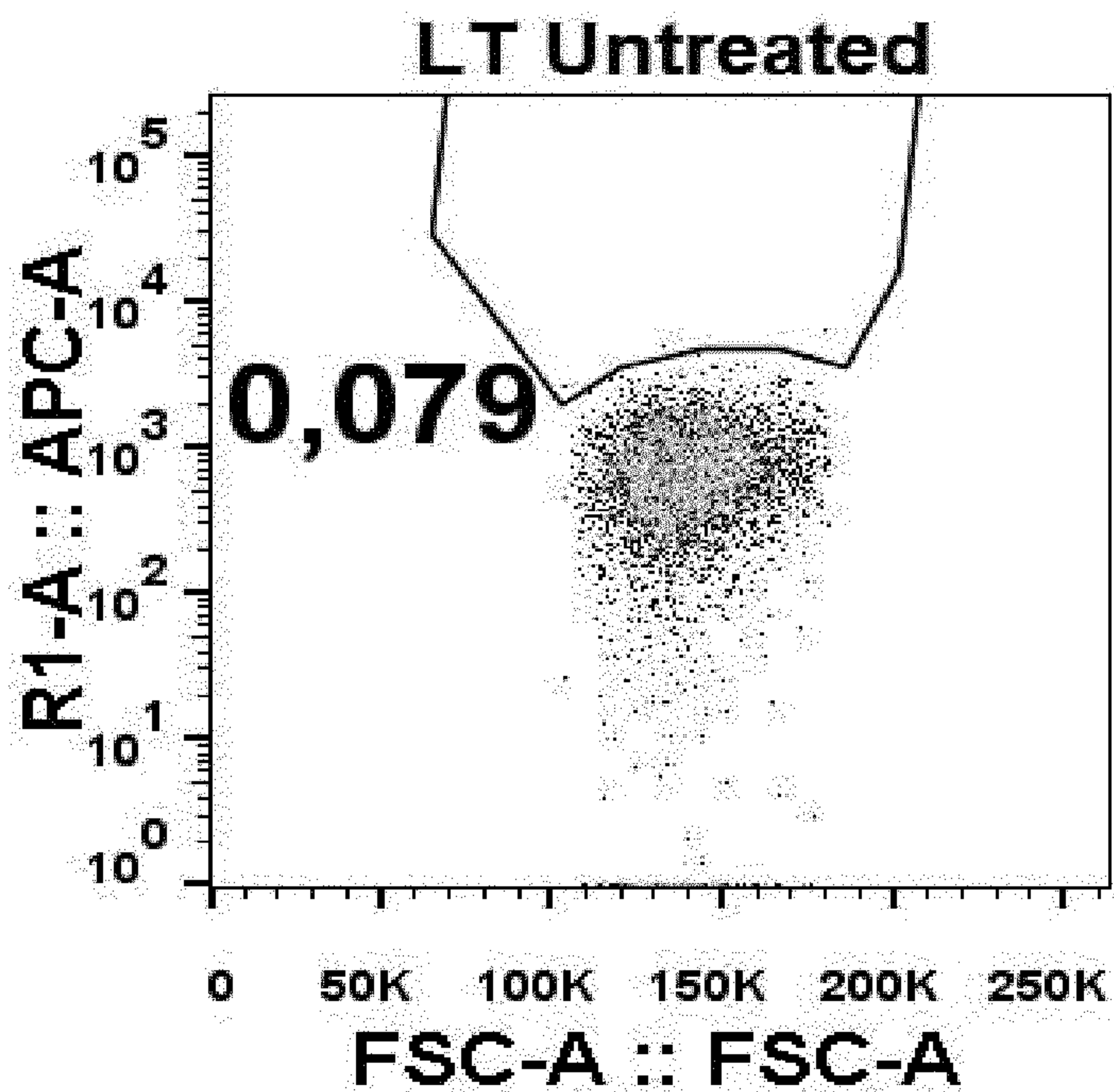

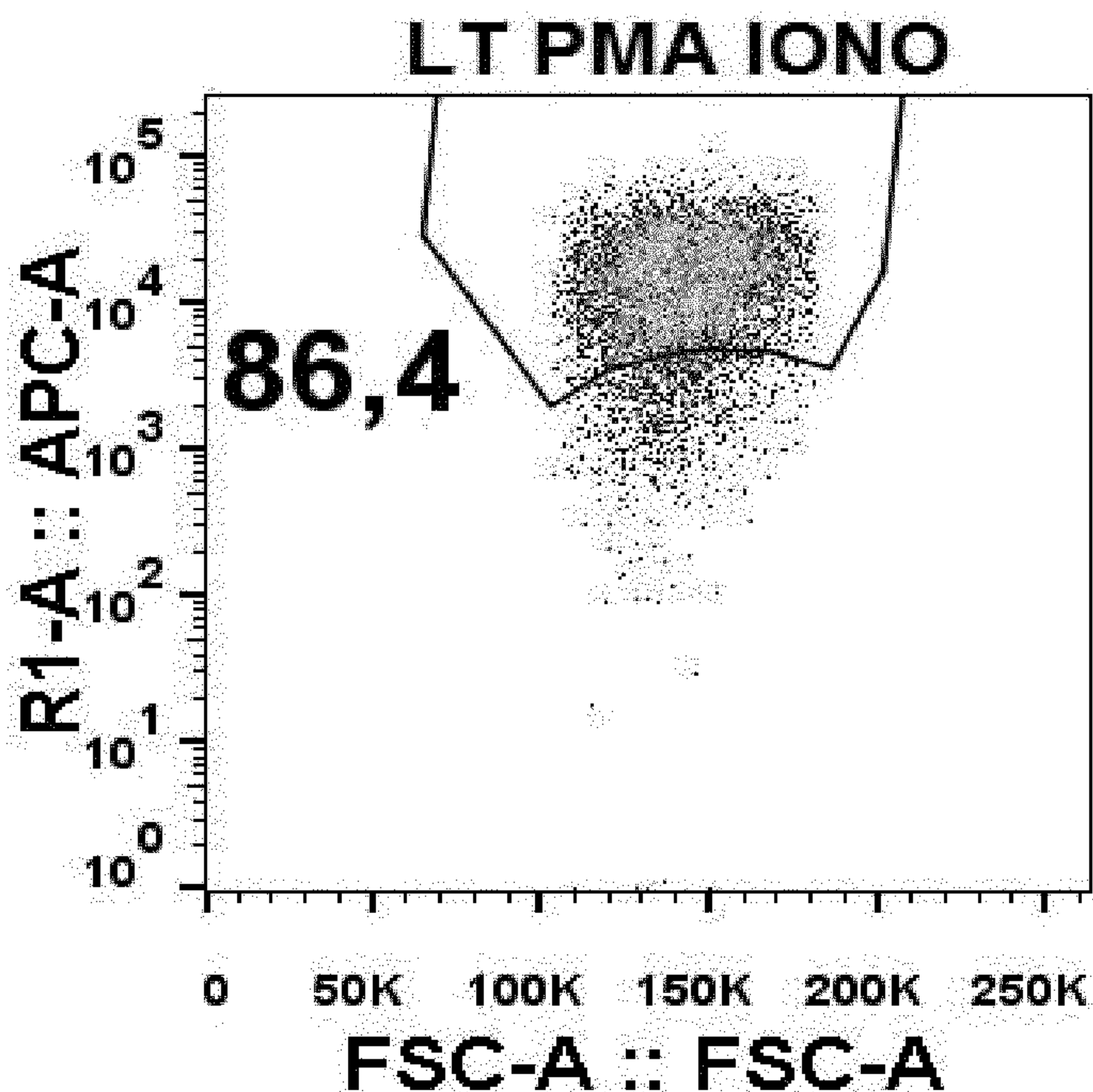

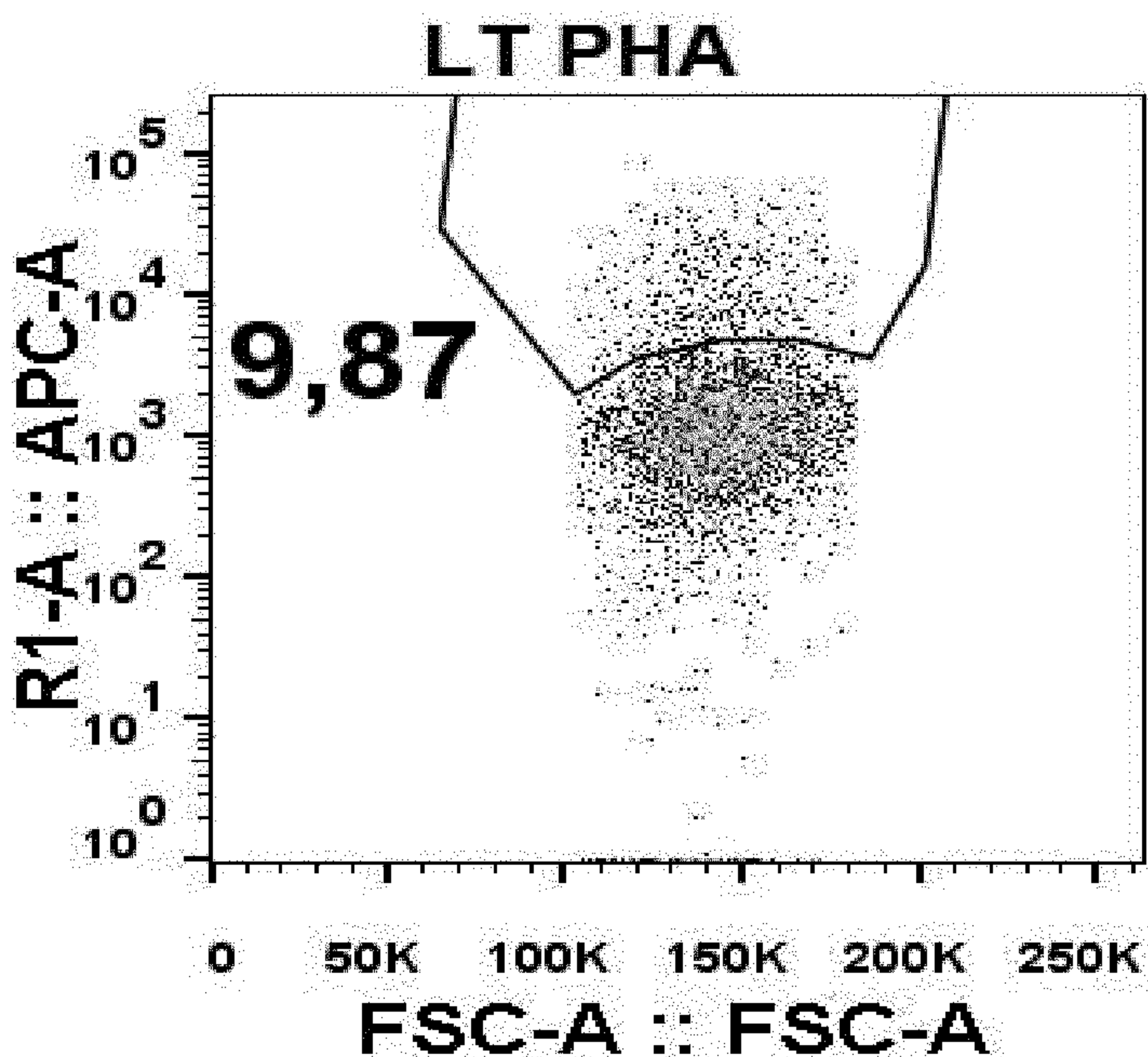

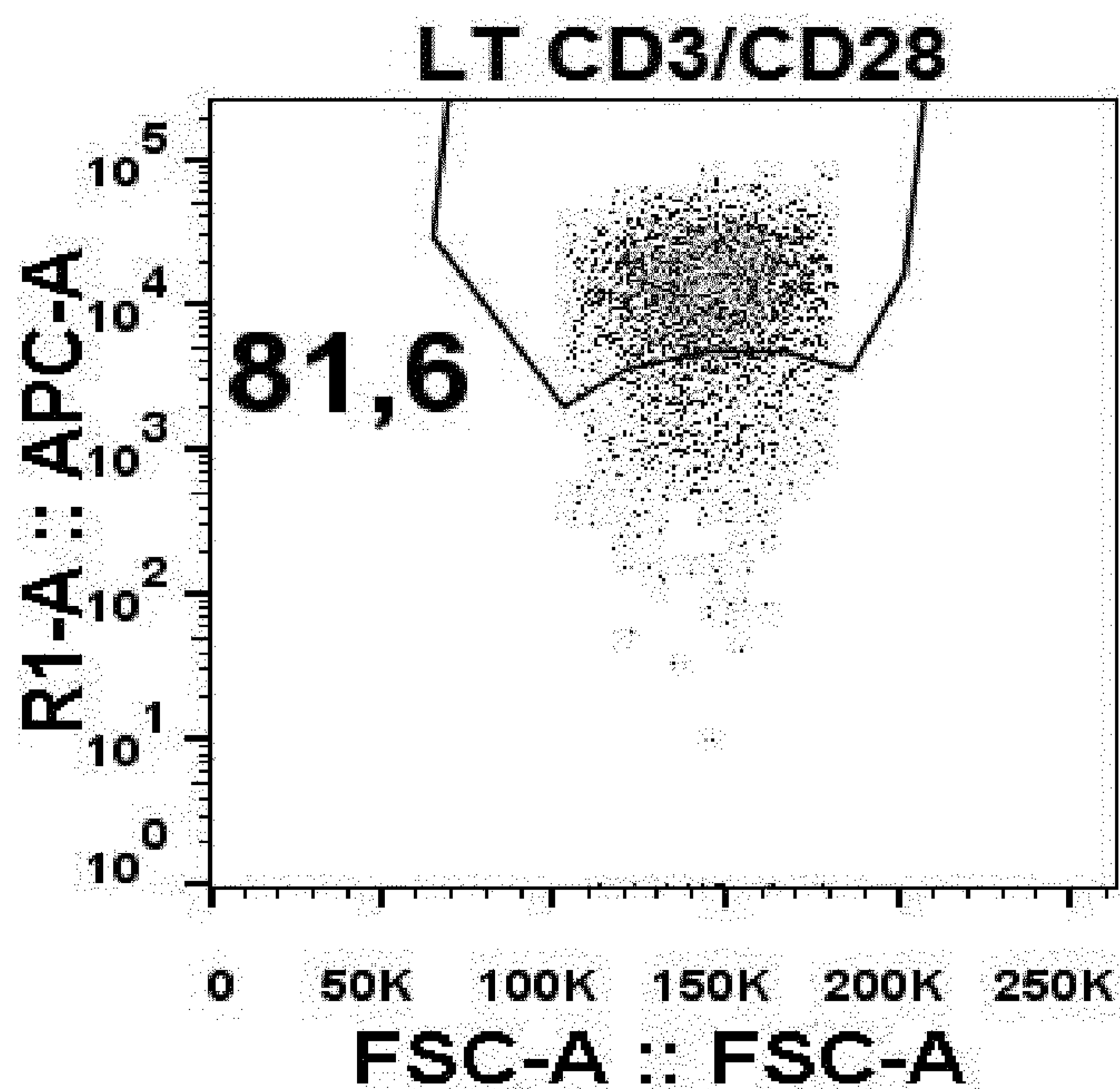

Figure 33 (cont.)

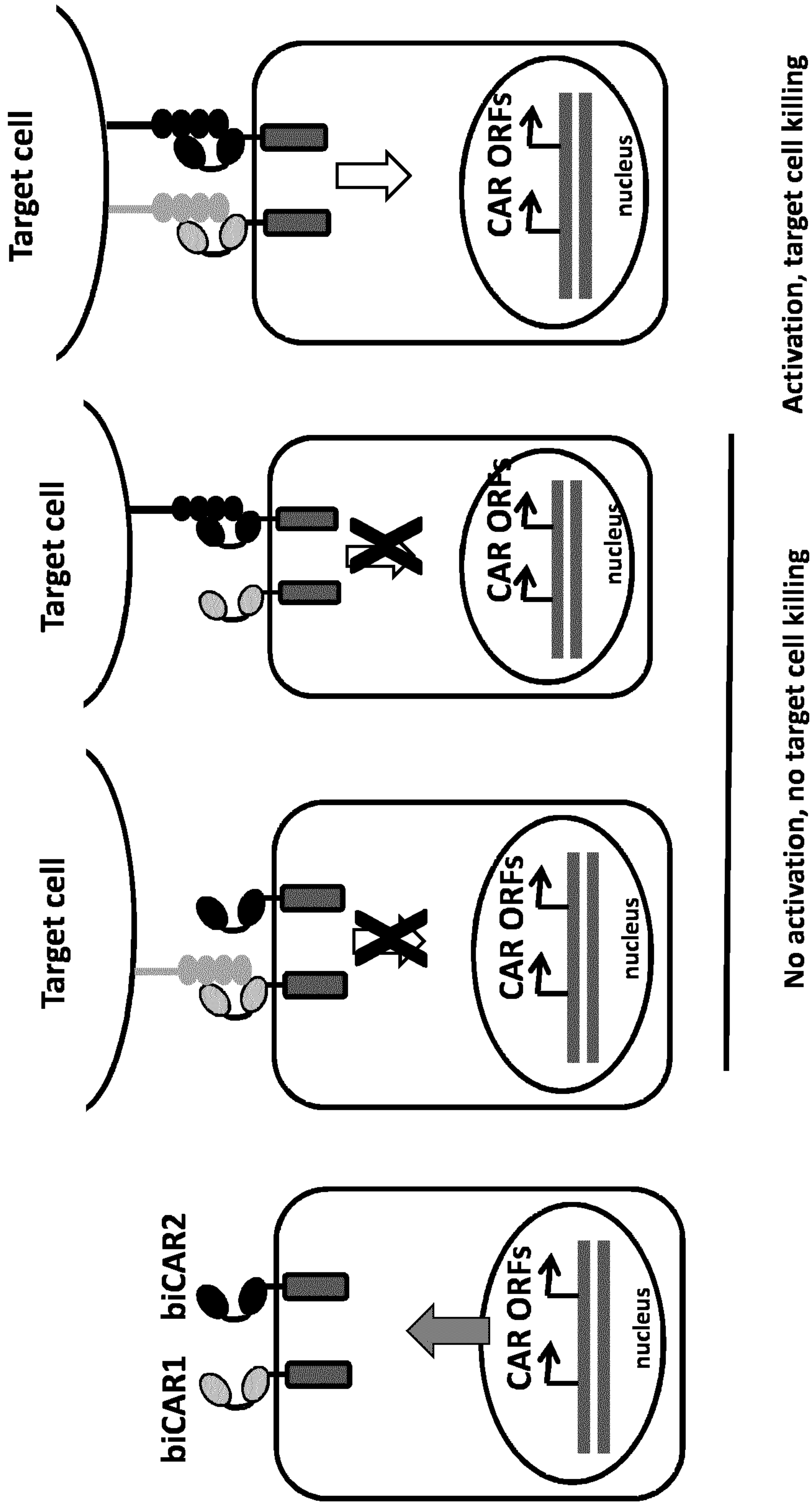

Fig. 34