	Inventaris Wob-verzoek W15-11								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS 2015125								
1	Aanvraagformulier				x		x	x	
2	Niet-technische samenvatting	x							
3	Projectvoorstel				x			x	
4	Flow chart			x					
5	Bijlage beschrijving dierproeven 1			x					
6	Bijlage beschrijving dierproeven 2			x					
7	Bijlage beschrijving dierproeven 3			x					
8	Bijlage beschrijving dierproeven 4			x					
9	Bijlage beschrijving dierproeven 5			x					
10	Bijlage beschrijving dierproeven 6			x					
11	Bijlage beschrijving dierproeven 7			x					
12	Overzicht aantallen			x					
13	Ontvangstbevestiging				x		x	x	
14	DEC-advies				x		x	x	
15	Mail aanvraag 22-6-2015				x		x	x	
16	Mail aanvullende informatie				x		x	x	
17	Aanvullende informatie				x		x	x	
18	Beschikking en vergunning				x		x	x	
19	Advies CCD		x						x

22 JUNI 2015

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

- U bent van plan om één of meerdere dierproeven uit te voeren.
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren. Of u geeft aan wat u in het vergunde project wilt wijzigen.
- Meer informatie over de voorwaarden vindt u op de website www.zbo-ccd.nl of in de toelichting op de website.
- Of bel met 0900-2800028 (10 ct/min).

1 Gegevens aanvrager

1.1 Heeft u een deelnemernummer van de NVWA?
Neem voor meer informatie over het verkrijgen van een deelnemernummer contact op met de NVWA.

☑ Ja > *Vul uw deelnemernummer in*	80102 (Hubrecht Instituut-KNAW) 80101 NIN
☐ Nee > *U kunt geen aanvraag doen*	

1.2 Vul de gegevens in van de instellingsvergunninghouder die de projectvergunning aanvraagt.

Naam instelling of organisatie	KNAW
Naam van de portefeuillehouder of diens gemachtigde	[redacted]
KvK-nummer	5 4 6 6 7 0 8 9

1.3 Vul de gegevens van het postadres in.
Alle correspondentie van de CCD gaat naar de portefeuillehouder of diens gemachtigde en de verantwoordelijke onderzoeker.

Straat en huisnummer	
Postbus	Postbus 19121
Postcode en plaats	1000GC Amsterdam
IBAN	NL94
Tenaamstelling van het rekeningnummer	Hubrecht Instituut / Nederlandshersen Instituut

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted] ☑ Dhr. ☐ Mw.
Functie	Group Leader
Afdeling	[redacted]
Telefoonnummer	[redacted]
E-mailadres	[redacted]

1.5 *(Optioneel)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	☑ Dhr. ☐ Mw.
Functie	
Afdeling	
Telefoonnummer	
E-mailadres	

1.6	*(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.	(Titel) Naam en voorletters	☐ Dhr. ☐ Mw.
		Functie	
		Afdeling	
		Telefoonnummer	
		E-mailadres	
1.7	Is er voor deze projectaanvraag een gemachtigde?	☐ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*	
		☑ Nee	

2 Over uw aanvraag

2.1	Wat voor aanvraag doet u?	☒ Nieuwe aanvraag > Ga verder met vraag 3
		☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.2
		☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.3
2.2	Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is?	☐ Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier
		☑ Nee > Ga verder met vraag 3
2.3	Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend?	☑ Nee > Ga verder met vraag 3
		☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6

3 Over uw project

3.1	Wat is de geplande start- en einddatum van het project?	Startdatum	0 1 _0 6 _ 2 0 1 5
		Einddatum	0 1 _0 6 _ 2 0 2 0
3.2	Wat is de titel van het project?	The molecular and cellular mechanisms of tumor initiation, growth, metastasis and thera	
3.3	Wat is de titel van de niet-technische samenvatting?	Het begrijpen van het ontstaan en ontwikkeling van kanker, uitzaaiingen en resistentie t	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	DEC-KNAW
		Postadres	[redacted] Amsterdam
		E-mailadres	[redacted]

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

- [x] Nieuwe aanvraag Projectvergunning € 741,00 Lege
- [ ] Wijziging € Lege

4.2 Op welke wijze wilt u dit bedrag aan de CCD voldoen.
Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.

- [ ] Via een eenmalige incasso
- [x] Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

- [x] Projectvoorstel
- [x] Niet-technische samenvatting

Overige bijlagen, indien van toepassing

- [x] flow chart
- [ ] table with experimental groups

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD of per post naar:

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.6). De ondergetekende verklaart:

- dat het projectvoorstel is afgestemd met de Instantie voor Dierenwelzijn.
- dat de personen die verantwoordelijk zijn voor de opzet van het project en de dierproef, de personen die de dieren verzorgen en/of doden en de personen die de dierproeven verrichten voldoen aan de wettelijke eisen gesteld aan deskundigheid en bekwaamheid.
- dat de dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU, behalve in het voorkomende geval de in onderdeel F van de bijlage bij het bij de aanvraag gevoegde projectvoorstel gemotiveerde uitzonderingen.
- dat door het ondertekenen van dit formulier de verplichting wordt aangegaan de leges te betalen voor de behandeling van de aanvraag.
- dat het formulier volledig en naar waarheid is ingevuld.

Naam	[redacted]
Functie	Directeur Instituten KNAW
Plaats	Amsterdam
Datum	0 1 - 0 6 - 2 0 1 5
Handtekening	[redacted]

Centrale Commissie Dierproeven

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80102
1.2	Provide the name of the licenced establishment.	KNAW
1.3	Provide the title of the project.	The molecular and cellular mechanisms of tumor initiation, growth, metastasis and therapy resistance

2 Categories

2.1 Please tick each of the following boxes that applies to your project.

- ☒ Basic research
- ☐ Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or
- ☐ Research aimed at preserving the species subjected to procedures
- ☐ Higher education or training
- ☐ Forensic enquiries
- ☐ Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

Cancer is one of the most deadly diseases in the western-world. When diagnosed early, a primary tumor can be surgically removed, and most patients can be cured at this early stage. However when cancer is diagnosed at a later stage, there is the chance that cells from the primary tumor have detached, spread to other sites in the body, and have

formed new distant tumor sites. At this stage cancer is far more difficult to treat and most patients die as a consequence of complications resulting from metastasis.
Upon genetic mutations, tumor cells “high-jack” cellular processes, that under non-disease circumstances, only takes place in different cell types or in specific phases of development. Examples of cellular processes that cancer cells “high-jack” include cell division and cell growth, a change from an epithelial cell state to a mesenchymal cell state (known as epithelial-to-mesenchymal transition (EMT)), and enhanced cell motility. The micro-environment (the environment that directly surround the mutated cell) and the macro environment (the whole tumor/animal) is an additional driver for picking the processes that are “high-jacked” by cancer cells. The acquisition of these molecular and cellular processes drives tumor initiation, growth, and metastasis.
There are many different cellular processes that can be “high-jacked” leading to cancer or leading to the progression of cancer. Different tumor types (e.g. breast vs colon) but even various variants of the same tumor type can have different genetic mutations and micro- and macro-environments, and therefore can adapt a different set of molecular and cellular processes that they up- or down-regulate. This also means that e.g. breast tumor cells behave differently than colorectal tumor cells, and that these tumor types need to be treated as two independent diseases that need to be investigated independently in their natural orthotropic environment in the context of a whole animal. This type of knowledge is key for the design of therapies that are tailor-made for every patient (personalized medicine) that aim to target the specific processes acquired by the tumor cells in that particular patient.
Tumors are extremely heterogeneous and consist of a variety of tumor cells that have “high-jacked” different processes due to a variation in mutations and environments that the cells experience. Sometimes, just a few individual cells high-jack cellular processes important for e.g. metastasis, while all the other cells have not acquired these traits. The behavior of these few dangerous cells cannot be studied with traditional techniques including histochemistry, (q)PCR and western blotting, since they provide a snapshot of a large population of cells and lack crucial information on the history of these few individual cells. The [redacted] lab has developed unique [redacted] to visualize and study the behavior of individual cells in living mice. Using these techniques, the [redacted] lab can identify and characterize the dynamic behavior of individual cells that are responsible many of the key processes of cancer. With these techniques, we provide unique insights in the molecular and cellular processes that play a role in tumor initiation, tumor progression, metastasis, and the development of therapy resistance. With this knowledge we have the ultimate aim to contribute to the improvement cancer diagnosis, cancer prevention, and cancer treatment in human patients.
Below it will be explained why it is important to study processes required for tissue homeostasis and development in the initiation of a tumor, how tumors develop and progress to a metastatic state and how the micro- and macro-environment is key in these processes.

1) Tumor initiation:
As already indicated above, for the initiation and progression of a cancer, multiple genetic modifications have to occur in the same single cell leading to “high-jacking” of processes specific for other cell types or developmental processes. It has been speculated that long-living cells are more likely to accumulate genetic lesions than short-living cells. In particular adult stem cells, that are located in a special microenvironment (stem cell niche) that provides cues to self-renew, are long-lived and are the source for all differentiated cells in the tissue. Therefore, it is hypothesized that accumulation of genetic oncogenic alterations in stem cells initiates neoplastic growth. Indeed, deletion of the tumor suppressor gene APC in intestinal stem cells leads to adenoma formation in the small intestine while deletion of this gene in differentiated cells does not. Recently, the idea that stem cells represent a static and long-living population of cells has been disputed. For example, we have developed a novel approach for continuous [redacted] stem cells in the gut ([redacted]), and showed that stem cells compete for the stem cell niche, so that the progeny of one stem cell may outcompete (all) other stem cells. Moreover, we showed that more differentiated cells can still revert back to a stem cell state when they enter the stem cell niche. Based on this it has been hypothesized that due to stem cell competition, stem cells with tumor-inducing mutations can be replaced by intact stem cells, thereby protecting tissues from initiating tumors when mutations are acquired. Therefore, the knowledge obtained from this type of experiments is important to fully understand how stem cells can accumulate mutations that are required for the initiation of cancer.

2) Cancer progression.
It is hypothesized that cell hierarchy (where just a few cells with stem cell properties drive growth) may also exist in tumors, referred to as the cancer stem cell hypothesis. However, both the actual existence of cancer stem cells as well as the similarities between cancer stem cells and (tissue) stem cells in other tissues are subject to scientific debate. Nevertheless, if correct, these cancer stem cells may also be long-lived (compared to their differentiated counterparts). Therefore these cells may also be susceptible for the accumulation of mutations that are required for a tumor to progress into a metastatic phenotype. These cancer stem cells may appear to be the driving forces of growth of the primary tumor and the metastases. Therefore, the cells that escape from the primary tumor and form distant metastasis should either be a cancer stem cell or cells that temporally “high-jack” these traits. To fully understand how tumors are growing and the state that cells should adapt to metastasize, it is of utmost importance to study how cancer stem cells behave during cancer progression.

3) The tumor microenvironment:
An expanding body of work shows that in addition to the intrinsic characteristics of tumor cells, the tumor microenvironment is a key determinant for angiogenesis (formation of blood vessels), growth, differentiation or

metastasis. The microenvironment of a tumor consists of at least four broad categories of factors including, 1) tumor cells, 2) non-tumor cells (e.g. myeloid, lymphocytes, fibroblasts), 3) secreted soluble factors, 4) non-cellular structural factors (e.g. extracellular matrix (ECM)). Tumor microenvironments are spatially and temporally diverse, and may direct different tumor behaviors. To fully comprehend tumor initiation and progression, it is key to study also the microenvironment that drives cellular behavior.

Research of our lab:
Over the years, we have significantly contributed to a better fundamental understanding of cancer initiation and progression. Our current research focused on the following questions: 1) how is cancer initiated, 2) how do tumors grow and progress, 3) how do tumors metastasize, 4) how become tumors resistant to therapy

3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

Over the years, we have developed state-of-the art intravital imaging techniques that enable us to study the dynamic behavior of individual tumor cells that have "high-jacked" different cellular processes than the tumor cells that surrounds them. The visualization of tissues with subcellular resolution in living mice gives us the ability to study individual cells and the ability to study the dynamic aspects of cancer that cannot be studied by any other means. Using our unique tools, it is our ultimate aim is to better understand how cancer is initiated, how cancer progresses and use this knowledge as a starting point on how cancer can be best treated in order to open avenues for the development of new and better cancer treatments.
Our intravital imaging studies focus on the following key questions:

1) What are the molecular and cellular processes important for tissue development and homeostasis, and what goes wrong when tumor growth is initiated?
2) What are the molecular and cellular mechanisms processes that play a role in tumor growth and progression?
3) What are the molecular and cellular mechanisms that play a role in the initiation and development of metastasis?
4) What goes wrong when current clinical strategies fail (therapy resistance, adverse effects of tumor injury), and how to improve this?

There are several reasons why we think that we can achieve our aims:
Our group is embedded in [redacted], which is a center-of-excellence on developmental biology, and stem cell and cancer research. [redacted] provides core facilities for various high-end techniques such as deep sequencing, histology, fluorescent imaging, mRNA expression array, flow cytometry. Moreover, the Hubrecht Institute has just renovated their animal facility, and now it can compete with the best animal facilities that can be found internationally. Dedicated staff provides the regular housing of the animals and support and counsels the scientist in their experiments. Within our group, we have one dedicated and very experienced scientist that overlooks all breeding of mouse lines, experiments and procedures, and trains new people when required. This guarantees that only trained and experienced people perform experiments.
Our research and experiments are constantly evaluated within our group, and by various other groups within our institute and campus. Moreover, our research is positively judged by national and international funding agencies including the [redacted]. Moreover, our group is member of [redacted], which is a consortium of prominent cancer research groups from seven research institutions in the Netherlands. Our ambition is to significantly improve life expectancy and quality of life for cancer patients and to provide multidisciplinary training for the next generation of cancer researchers and specialists. The scientists working in our group are selected based on their excellence and their commitment to the mission of the program.
Over the last few years, we have built up a repertoire on of state-of-the-art [redacted] techniques to study the molecular and cellular aspects of cancer initiation and cancer progression, and cancer treatment in a unique way. In addition a wide range of new reporter mice have been generated and used. This has led to many new discoveries and breakthroughs published in high ranked journals (e.g. Nature and Cell) and our research has been awarded with international prices (e.g. stem cell young investigator award). Our research is funded by all major funding agencies, and received the most prestigious grants (e.g. ERC consolidator).
Our embedment in an excellent scientific environment, our unique techniques and approaches, and our previous achievement makes it very likely that with the experiments described in this project we will make large contributions to our main research questions.

3.3 Relevance

What is the scientific and/or social relevance of the objectives described above?

Tumors are extremely heterogeneous where the individual cells can “high-jack” molecular and cellular processes that the original healthy cell did not have yet. Our research, and especially our intravital imaging technique, has the unique potential to study these individual cells to reveal the molecular and cellular processes that are acquired and the effect it has on their behavior. Therefore, it is expected that this work will provide totally new insights how cancer is initiated, how cancer progresses, how tumors metastasize, and why current treatments are not sufficient/optimal. This fundamental knowledge is required for the development of novel and/or improved therapies against cancer. Moreover, in addition to the field of cancer, this fundamental knowledge is also important for other fields including fundamental cell biology.

3.4 Research strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

For our overall design of the project, see the flow Chart in attachment 1.

Based on data from previous experiments and available literature, we generate a hypothesis or question about the molecular and/or cellular mechanism of initiation, progression or therapy resistance of cancer. The questions/hypothesis will first be carefully tested on patient material by e.g. immunohistochemistry. For example, we ask whether cells in the tumor can adapt a mesenchymal state in addition to the epithelial state (EMT). Although immunostaining can reveal the existence of cells with these types of states, it only draws a static picture of tumors and can, for example, not show whether epithelial cells adapt only temporally a mesenchymal state. When we have these types of questions/hypotheses that cannot be answered in human material alone, experiments will be designed in cell lines and/or organoids (3D cultures of human or mouse primary cells). For example, we can test whether epithelial cells can temporally adapt a mesenchymal state by exposing these cells to growth factors. As explained in the background, in vitro conditions lack the full complexity of the in vivo environment, and therefore do not tell the full story. For example these experiments do not show whether the temporal mesenchymal state is crucial for successful metastasis when cells need to adapt to a new microenvironment. To answer these kinds of questions mouse experiments will be considered. We will set up optimal breeding schemes to minimize the number of mice to get the correct complex genotype. Moreover, during the experiments mice will be monitored extensively to detect and avoid unnecessary discomfort. For experiments, existing mouse lines will be used, and if required, we will generate a new mouse line(s).

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

See also the flow chart in attachment 1. In the red boxes the choice of animals (A to C), interventions (I to IV) and readout (1 to 3) is indicated and which is described below.

When we have decided to perform an animal experiment, the experiment is design is based on three choices:
A) Choice of mouse model
B) Choice of type of intervention
C) Choice of readout and end-point of the experiment

The considerations of the choices will be explained in more detail below.

For research questions 1, we need to compare normal tissue homeostasis/development with tumor-initiating tissue homeostasis/development. For research question 2 to 4 (see 3.2 purpose), mice need to develop tumors. For 70% of these experiments, tumors are induced (overexpression of oncogenes, or depletion of tumor suppressor genes) and 30% by transplanting neoplastic tissues or cells. As experimental read out, we analyze tissues ex vivo or by intravital microscopy. At the end point of intravital microscopy, tissues will always be analyzed ex vivo to reduce the number of mice required for 3.4.4.1. These experiments are described in the appendices 3.4.4.1. 3.4.4.2, 3.4.4.3, 3.4.4.4, 3.4.4.5. and 3.4.4.6.

All experiments described in appendices 3.4.4.1 to 3.4.4.6 can provide knowledge on the key questions 1 to 4 (see 3.2.), and the decision route in the flow chart is determined by e.g. the studied tumor model. An example of a typical experiment is to study the migration behavior of cancer cells that have “high-jacked” stem cell properties. For breast tumors, we will choose a mouse model in which an oncogene is overexpressed (e.g. Choice B II for intervention to

overexpress PyMT, and choice C 2 for read-out). To answer the same question for colorectal tumors, a different decision route is required. In this case, the tumor suppressor gene APC depletion leads to tumor formation throughout the colon and subsequently to a non-functional intestine, and a human end point is reached before the tumor progresses to a stage where tumor cells have acquired stem cell properties. In this case, a small piece of colon from APC depleted mice will be transplanted into a recipient mouse (choice B III) so that only one tumor is formed leaving the intestine functional. Since the mouse does not reach the human end point at early stages of tumor progression, the tumor can progress to a stage where tumor cells can “high-jack” stem cell properties and the measurement can be done (choice C2). Thus for the same research questions, different decision routes need to be taken depending on the tumor model and required intervention.

Generation of GM mice (appendix 3.4.4.7):
We will generate new mouse line(s) via standard oocyte injection, blastocyst injection, or via the Crispr/Cas9 system. The Crispr/Cas9 system will especially be used as highly efficient tool for simultaneously multi-gene editing. This prevents the generation and breeding of multiple homozygotes from individually targeted ES cells (Reduction of the 3Rs).

Next, we will identify a possible hampered phenotype in novel (compound) mouse models according to the Consensus document on genetically altered animals. Therefore new transgenic lines and/or KO lines generated via classical methods and/or novel combinations and used for breeding of these aforementioned lines will be monitored for 2 generations to determine the absence or presence of constitutional discomfort.

For some transplantation experiments (e.g. when human organoids are transplanted), immune deficient acceptor mice are required. We breed our own immune deficient NOD-SCID mice. For this, we have 6-10 breeding pairs that will be replaced every 6 month that leads to offspring that will be used for experiments. According to the working document on genetically altered animals of the National Competent Authorities for the implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes (corrigendum of 24 Jan. 2013) breeding of immune deficient mice even when kept under proper barrier conditions is considered an experiment and the breeding of these animals is described in 3.4.4.7.

A. Mouse models:

There are several considerations in choosing an animal model:
- GMM that expressed a fluorescent marker in a specific cell type/lineage
- GMM that changes expression of a functional gene(transgene knockout)
- Spontaneous tumor model
- Immune-deficient/wt recipient mouse
- Wt

The choice will be on the following considerations:
- Type of tumor: Tumor cells can “high-jack” a wide variety of molecular and cellular processes, and not one tumor is alike. Therefore it is important to investigate different tumor types and various variants of the same tumor type to reveal whether the acquisition of a particular molecular and cellular process is a general or specific phenomenon (personalized medicine).
- Readout parameters

If the required GMM is not available, than we will obtain this model by generating, importing or by crossing existing models (3.4.4.7).

B. Interventions:

Apart of I) ‘no intervention’ we will use the following additional groups of interventions: II) upregulation or reduction cell types or expression of genes by genetic approaches, drugs or injury, III) transplantation of tissue or cells, and IV) a combination of intervention II and III in the same animal.

I) No experimental interventions (3.4.4.1 and 3.4.4.2):
To visualize for example how stem cells behave during tissue development, we analyze ex vivo (3.4.4.1) or intravital image (3.4.4.2) reporter mice in which e.g. stem cells are labelled with GFP.

II) Cell type specific gene (in)activation and (over)/(mis)expression (3.4.4.1 and 3.4.4.2):
To identify for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating these processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze GM mice in which the gene(s) will be

activated, inactivated, overexpressed and/or misexpressed.

In contrast to the conventional gene-targeting strategy, the use of e.g. the Cre/LoxP recombination system in conjunction with gene targeting has greatly expanded the versatility and avenues with which biologic questions can be addressed in the mouse. This system allows us, by strategically incorporating Cre recombinase recognition (LoxP) sites into the genome and the subsequent expression of the Cre recombinase, to study the consequence of specific ablation, activation and/or over/misexpression of a specific protein. In particular, when Cre is expressed in mice harboring a LoxP-containing target gene, the desired gene modification can be restricted to certain developmental stage, organ (e.g. intestine), cell type (e.g. stem cell), pathogenesis (e.g. cancer cells) of the mouse depending on the specificity and timing of recombinase expression. This system will be used to manipulate molecular and cellular events, but also to induce an early stage of tumor formation by depleting tumor suppressor genes (e.g. APC in the intestine).

Moreover, the introduction of novel gene(s) will help us in further characterizing the role of expressing cells. E.g. the introduction of fluorescent markers in (putative) different cancer cells pools (e.g. cancer stem cells vs more differentiated tumor cells) allows the isolation of these pools of cells by flow cytometry. Upon isolation, the different pools of cells can be characterized by e.g. gene expression profiling. Moreover, the (combined) introduction of e.g. an exogenous toxin receptor in the same cells allows us to specifically kill these cells upon the administration of the toxin to the mouse, allowing us to determine the consequence of this cell depletion during development and/or in tissue homeostasis. This is also true for over or mis- or overexpression of (mutated) genes, especially oncogenes to induce tumor formation (e.g. the PyMT oncogene in breast tissues).

Administering small molecule compounds/drugs/toxin/chemical or control substances (e.g. inhibitors or agonists of specific pathways), we might be able to rescue or mimic the phenotypes of the in vivo genetic deletions and/or activations and therefore further identify the function of these cells in vivo. If possible and/or relevant, we will always test these small molecule compounds/drugs/chemicals first on in vitro growing organoids and in case relevant effects are observed shift to in the in vivo models.

In a small number of animals (<3%), injuries will be used to manipulate for example the recruitment of cell types (e.g. immune cells) with subsequent effects on expression profile of different cells in the tissue. For instance in the past we studied how the retrieval of biopsies induces an injury that recruits immune cells that subsequently release chemokines that induce metastasis.

III) Transplantation tissues/cells/organoids (3.4.4.3 and 3.4.4.4):
To identify the role of various cell types on developmental and cancer processes, we need to transplant (genetically modified) human and murine tissues, cell or organoids in mice. For example, in the past we have transplanted colorectal organoids (3D cultures) in mice to induce colorectal tumor growth. Moreover, we have transplanted red-labelled erythrocytes to long-term label blood vessels in order prevent a more discomfort causing procedure of daily injections of fluorescent-dextran enabling only short-term labeling of blood vessels.

IV) In some cases the transplantation should be combined with the cell type specific gene (in)activation and (over)/(mis)expression) (3.4.4.5 and 3.4.4.6):

The cumulative level of discomfort is dependent on both the intervention (moderate) and the read-out. Therefore for both the ex vivo (3.4.4.5) and intravital imaging (3.4.4.6), the cumulative discomfort is moderate.

Readouts/endpoint
We have three types of readouts:
1) Ex vivo analysis (appendices 3.4.4.1, 3.4.4.3 and 3.4.4.5),
2) Acute Intravital imaging (appendices 3.4.4.2, 3.4.4.4, and 3.4.4.6)
3) Chronic imaging (appendices 3.4.4.2, 3.4.4.4, and 3.4.4.6).

To study the molecular and/or cellular mechanisms of cancer processes, we need to analyze histologically and molecularly the tissues and cells from the mice ex vivo, and established cells lines and organoids (3D culture system) to perform in vitro studies. These cell lines and organoids will enable us to perform some of the studies in vitro. We might also use these cell lines and organoids to generate new tumors upon transplantations.

To study dynamic processes that are missed in static histological images, in vivo imaging will be performed. For the imaging experiments two different strategies will be used: (a) Imaging in an acute experiment under anaesthesia and (b) Implanting an imaging window (such as the breast, skin, abdomen and skull) and repeatedly image through that window. For long-term studies (>24hrs) the animal needs to be imaged by multiple imaging sessions, and therefore imaging windows are required. For some research questions, the tissue needs to be visualized frequently for a relative short-period of time (max 3 days). It is not an option to anesthetize the animal multiple times a day, so the animal will be constantly anesthetized. In this case, an imaging window is not required and an acute imaging experiment will be chosen. We have a lot of experience in the lab with these procedures, and only well trained researchers will perform these experiments.

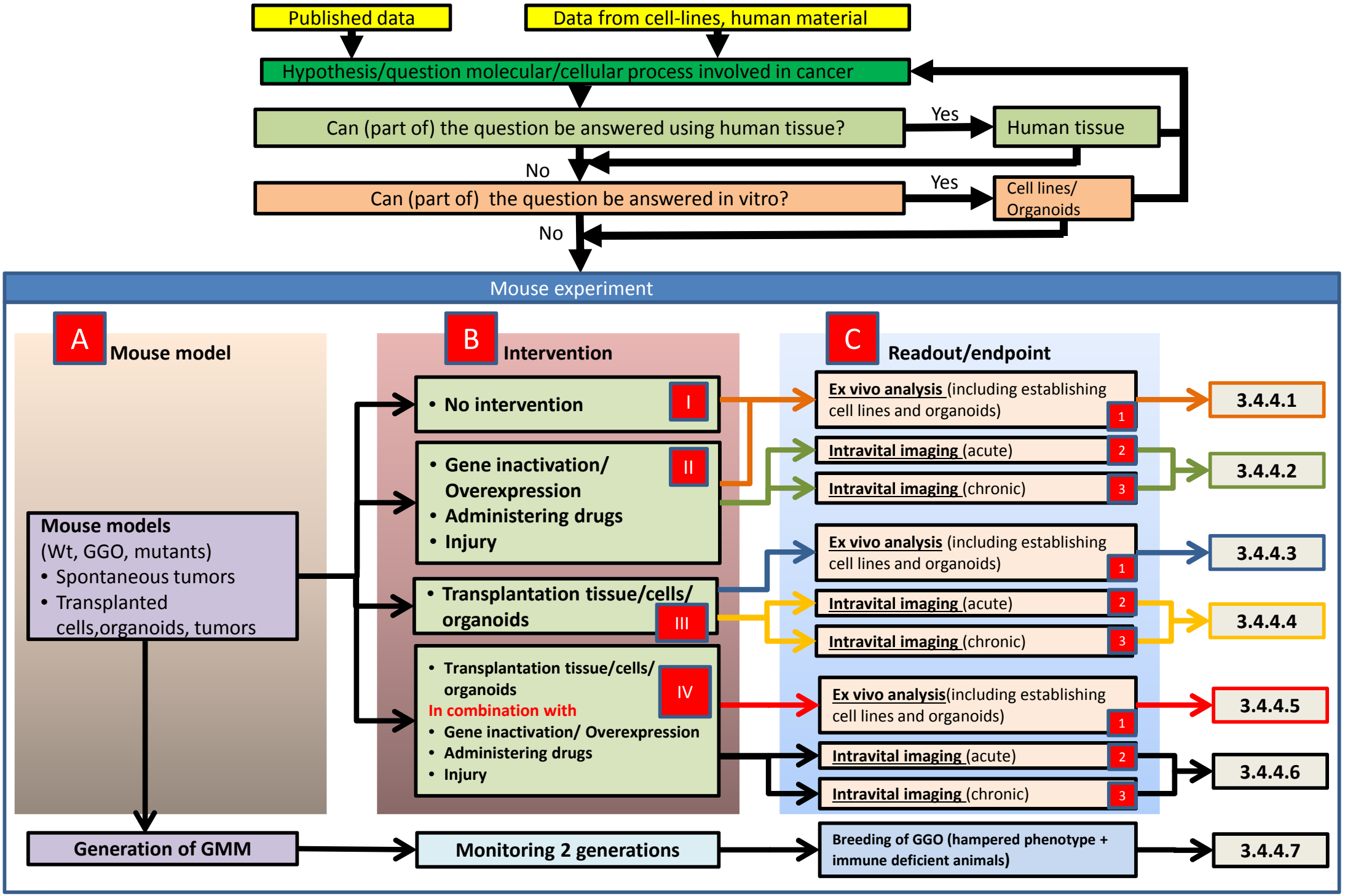
3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points.

Whenever possible, we will perform pilot studies with the minimum amount of animals possible. We will only use well-established reagents and protocols to induce expression or deletion of the candidate gene/s. For every experiment, we design the experiment with clear go-no-go decisions, to reduce the amount of cumulative discomfort and/or the number of animals. For every experiment, the best trade-off will be made. For example, for most experiments we first consider ex vivo experiments (mild discomfort), before we consider intravital imaging experiments (moderate discomfort). In some experiments we first consider intravital imaging, since either some questions can only be answered by imaging the same tissue over multiple imaging sessions, or it significantly reduces the number of required mice (can be up to a reduction of 20x); multiple time points can be measured in one individual, and there is no inter-mice variation. After completion of the intravital imaging experiments, cells, tissues and organs will be isolated and analyzed to reduce the number of mice required for 3.4.4.1, 3.4.4.3, and 3.4.4.5.

Where possible, mice with inducible alleles will be used, so mice will not display a phenotype before the induction of the alleles. Experiments will be done sequential. When mice show signs of discomfort (e.g. appearance of a tumor), the mouse will be sacrificed and not used for breeding anymore. If homozygous mice doesn't show any discomfort we will keep the mice on a homozygous background, thereby reducing the number of mice.

3.4.4 List the different types of animal procedures. Use a different appendix 'description animal procedures' for each type of animal procedure.

Serial number	Type of animal procedure
1	Gene (in) activation interventions (ex vivo analysis)
2	Gene (in) activation interventions (in vivo imaging)
3	Transplantation (ex vivo analysis)
4	Transplantation (in vivo imaging)
5	Transplantation, gene (in) activation interventions (ex vivo analysis)
6	Transplantation, gene (in) activation interventions (in vivo imaging)
7	Generation, welfare assessment and breeding GMM
8	
9	
10	



Indicates choice (A-B), type intervention (I to IV) ,and read-out (1 to 3) as decribed in the project proposal 3.4.2.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80102
1.2	Provide the name of the licenced establishment.	Koninklijke Nederlandse Academie van Wetenschappen (Hubrecht Institute)

1.3 List the serial number and type of animal procedure.

Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.

Serial number	Type of animal procedure
3.4.4.1	Ex-vivo analysis in mouse models and after gene inactivation/overexpression, administering drugs and injury

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general design of the experiments in this appendix is shown in the attached flowchart.
Our aim is to determine in ex vivo analysis the molecular/cellular mechanisms required for tissue homeostasis/development, tumor initiation, tumor progression, cancer diagnosis, cancer prevention, and cancer treatment via the analysis of (compound) mouse models.

The different components of the proposed experiments are (see also the flowchart in appendix 1):

A. <u>Mouse models:</u>

There are several considerations to choose for a specific animal model:

- Wt mice,
- Genetically modified mice that expressed a fluorescent marker in a specific cell type/lineage. In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to induce the expression of the fluorescent marker.
- Genetically modified mice that changes expression of a functional gene (transgene knockout). In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to change the expression of the functional gene
- Spontaneous tumor model
- Immune-deficient/wt recipient mouse

The choice will in all cases based on the combination of the following considerations:

- Aim/specific question (normal tissue development, tumor development, establishing cell

lines/organoids or a cell type specific expression of a fluorescent marker for an immunohistochemical /cell sorting/lineage study)

- Type of tumor
- Aim/readout parameters (e.g. static vs dynamic, long-term vs short-term dynamics)

To identify/validate for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze GM mice in which the gene(s) will be activated, inactivated, overexpressed and/or misexpressed. In some cases, mice get a special diet (but this does not lead to any discomfort).
We will use inducible systems including e.g. the well-known Cre-LoxP system in which a floxed gene can be deleted, overexpressed or misexpressed upon the induction of the Cre enzyme via the administration of tamoxifen. In this way the desired gene modification can be restricted to certain developmental stage, organ (e.g. intestine), cell type (e.g. stem cell). Successful deletion/activation via (e.g.) Cre enzyme induction might be monitored via expression of a reporter gene (e.g. LacZ or fluorescent protein(s)).

We will also use endogenous and exogenous promotors that are tissue or cell specific to drive expression of genes.
The presence of e.g. a fluorescent marker in a (putative) stem cell allows us to localize these cells by histology and to isolate the fluorescent expressing cells via FACS sorting, which allows us to analyse (gene expression profile) and culture these cells.
The introduction of e.g. a toxin receptor (e.g., Diphtheria toxin receptor) in the cells allows us to specifically kill these cells upon the administration of the toxin (e.g., Diphtheria toxin). This study allows us to determine the consequence of the loss of the toxin receptor expressing cells during development and for tissue homeostasis.
If the required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models (3.4.4.7).

B. No Intervention:

I. No experimental interventions:

To visualize for example how stem cells behave during tissue development, we analyze in ex vivo experiments reporter mice in which e.g. stem cells are labelled with GFP.

Interventions:

II Experimental interventions II:

-To identify/validate which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze GM mice in which functional gene(s) will be activated, inactivated, overexpressed and/or misexpressed.
-The administration of small molecule compounds/drugs/chemicals/toxins, we might be able to rescue or mimic the phenotypes of the in vivo genetic deletion(s) and/or activation(s) and therefore further identify the function of these cells in vivo.The mice might be injected with DNA labelling agents shortly before euthanasia to measure the proliferation capacity of the stems and their derivatives.
-Injuries (such as the surgical removal of a tumor, taking biopsies (not for the analysis, but for the injury), making a wound) will be made to investigate for example the recruitment of cell types (e.g. immune cells) with subsequent effects on expression profile of different cells in the tissue and the tumor.

C. Readout parameter

1. Ex vivo analysis

In all experiments, animals will be killed and embryos, neonatal and/or adult organs will be isolated for detailed analysis of the consequences of the genetic alteration and/or treatment on the (developing) tissue(s). Analysis will include among others histological sections labelled with antibodies or antisense RNA probes, RNA expression analysis, DNA or protein extracts. Also, cells from organs might be isolated

by FACS and/or cultured in vitro (organoids).

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

1. Tissue sampling for genotyping and identification via ear and tail biopsy resp. under anaesthesia (4% (isoflurane/oxygen).
2. Administration of transgene inducing or deleting agents or control substances alone or in combination, continuously or intermittently by one or more of the following routes:
   a) in diet or drinking water (max. 1 time, < 2 wks)
   b) subcutaneous (max. 3 time)
   c) intraperitoneal(max. 7 times)
   d) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia
   e) oral (max. 10 times)
3. Administration of small molecule compounds, drugs, toxin, chemicals or control substances alone or in combination, continuously or intermittently by one or more of the following routes:
   a) in diet or drinking water (max. time, <2 wks)
   b) subcutaneous (max. 10 time)
   c) intraperitoneal (max. 10 times)
   d) implantation of a slow release pellet subcutaneously under adequate anaesthesia and analgesia (1 time)
   e) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (1 time)
   f) oral (max. 10 times)
4. (Optional) Administration of a labelling agent (e.g. BrdU) via one of the following routes:
   a) intraperitoneal (max 3 times)
   b) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (1 time)
   c) intravenous (max 3 times)
5. (Optional) To mimic the injury resulting from e.g. surgical removal of a tumor or obtaining a biopsy, (tumor) tissues will be 'injured' under adequate anaesthesia and analgesia
6. All animals will be killed and organ(s)/tissue will be isolated for ex vivo analysis
   a) Adult mice: via CO2/O2 method or perfusion fixation under lethal dose of Nembutal.
   b) Embryo's and neonates (until P5): will be put on melting ice water for 10 min. (but not in contact with) after which they will be decapitated and the head immediately frozen (or the brains dissected and fixed).

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mus musculus, (Wt, genetically modified, mutants)
Origin: Hubrecht institute/external licensed breeders.
Embryos(> E13): max. 200
Neonates(until weaning): max. 100

Adult: max. 4000
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand tissue development/homeostasis and cancer in the context of the whole organism. For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

For the type of experiments described in this procedure, it is difficult to calculate the exact number of animals, since for various experimental setups different amount of mice are required, and the variation and outcome of the experiments are unknown. The number of mice required depends on the mouse model (e.g tumor incidence, tumor heterogeneity), the type of intervention and the characteristics of the measuring parameters and can therefore only be described in global and strategic terms. For these reasons we have provided a total number of mice based on experience over the past 5 years with these types of experiments in our group. Before we will start an experiment we will write an application to the IVD. In this application we will exactly describe which considerations, facts and results have led to the proposition of the number of animals needed for these experiments.
We have estimated the number of animals based on experience over the past 5 years. To give an idea of typical experiments we have done in the past 5 years:

1) Animals (including embryos, neonates and adults) are sacrificed at various developmental stages without experimental intervention. To examine e.g. the histological morphology of a stage, typically 6 mice are required per time point, and typically 5 time-points are taken. For example such a typical experiment requires 30 mice. On average, we had typically 8 of this type of projects per year, so over a 5 year time span we expect to require approximately 40 of these typical experiments.
2) In the case of generating tumors in the intestine via the inactivation of a single gene (e.g. Apc) in the stem cells (via Lgr5-ires-creert) a maximum of 30 mice may be required (5 (number of mice per group) * 6 (different time points required to follow the development of intestinal tumors over time). On average, we had typically 6 of this type of projects per year, so over a 5 year time span we expect to require approximately 30 of these typical experiments.
3) Influence of e.g. drugs on development of intestinal tumors as described above in 2: Two groups of mice as described above in 2 are typically treated with vehicle and the drug. So 60 in total (30 for vehicle, 30 for the drug). On average, we had typically 7 of this type of projects per year, so over a 5 year time span we expect to require approximately 35 of these typical experiments.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Before we consider the import or generation and subsequently analysis of (compound) GGM to study cancer models, we first will extensively analyze cell lines, existing tissue patient material and/or organoids. However, animal studies are unavoidable if we seek for the fundamental insight into the

molecular and cellular mechanisms that drive tissue homeostasis/development, cancer initiation, growth and metastasis.

We make extensive use of human material and in vitro experiments using cell lines and organoid culture (3D cultures) where possible, which extensively reduces the animal numbers. The use human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum.

Whenever possible, we will perform pilot studies with the minimum number of animals possible.
Where possible, mice with inducible alleles will be used, so mice should not display a phenotype before the induction of the alleles.

Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.

If homozygous mice doesn't show any discomfort we will keep the mice on a homozygous background, thereby reducing the number of mice need for breeding.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 conditions.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

If required, isoflurane will be used general anaesthesia and Temgesic to relieve pain.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Other than in terminally anaesthetized animals dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that will result in no more than transient, light discomfort. In less than 3% of all experiments, pumps/pellets will be implanted which leads to a moderate discomfort.
Due to administration of inducing agents or other substances animals will be experiencing no follow up effects.
Animals bearing tumors will never reach end-stage clinical effects. The scientific endpoints of all studies are much earlier then the humane endpoints.
It is expected that no animals (0%) will be experiencing more then mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day.

Explain why these effects may emerge.

Tumor development and/or inflicted tissue damage due to genetic alterations and/or administration of small molecule compounds/drugs/chemicals/toxins.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort.
In all cases the guidelines of the Code of Practice Animals in Cancer research will strictly be followed

Indicate the likely incidence.

Expected 0% within time frame of the experiments.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Embryo's(>E13): mild 100%
Neonates: mild 100%
Adult: mild 96%, moderate 1% <1day, moderate <3% constantly due to implantation pellet/pump

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed to use their organs in ex vivo analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
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- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80102
1.2	Provide the name of the licenced establishment.	Koninklijke Nederlandse Academie van Wetenschappen (Hubrecht Institute)

1.3 List the serial number and type of animal procedure.

Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.

Serial number	Type of animal procedure
3.4.4.2	In vivo imaging in mouse models and after gene inactivation/overexpression, administering drugs and injury

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general design of the experiments in this appendix is shown in the attached flowchart.
Our aim is to determine with in vivo imaging the molecular/cellular mechanisms required for tissue homeostasis/development, tumor initiation, tumor progression, cancer diagnosis, cancer prevention, and cancer treatment via the analysis of (compound) mouse models.

The different components of the proposed experiments are (see also the flowchart in appendix 1):

A. <u>Mouse models:</u>

There are several considerations to choose for a specific animal model:

- Wt mice,
- Genetically modified mice that expressed a fluorescent marker in a specific cell type/lineage. In some cases transgene inducing or deleting agents (e.g. tamoxifen) are administered to induce the expression of the fluorescent marker.
- Genetically modified mice that changes expression of a functional gene(transgene knockout). In some cases transgene inducing or deleting agents (e.g. tamoxifen) are administered to change the expression of the functional gene.
- Spontaneous tumor model
- Immune-deficient/wt recipient mouse

The choice will in all cases based on the combination of the following considerations:

- Aim/ specific question (normal development, tumor development, establishing cell lines/organoids or a cell type specific expression of a fluorescent marker)
- Type of tumor
- Aim/readout parameters (e.g. static vs dynamic, long-term vs short-term dynamics)

To identify/validate for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze GM mice in which the gene(s) will be activated, inactivated, overexpressed and/or misexpressed. In some cases, mice get a special diet (but this does not lead to any discomfort).
We will use inducible systems including e.g. the well-known Cre-LoxP system in which a floxed gene can be deleted, overexpressed or misexpressed upon the induction of the Cre enzyme via the administration of tamoxifen. In this way the desired gene modification can be restricted to certain developmental stage, organ (e.g. intestine), cell type (e.g. stem cell). Successful deletion/activation via (e.g.) Cre enzyme induction might be monitored via expression of a reporter gene (e.g. LacZ or fluorescent protein(s)).

We will also use endogenous and exogenous promotors that are tissue or cell specific to drive expression of genes.
The presence of e.g. a fluorescent marker in a (putative) stem cell allows us to localize these cells by in vivo imaging and to isolate the fluorescent expressing cells after the experiment via FACS sorting, which allows us to analyze (gene expression profile) and culture these cells.
The introduction of e.g. a toxin receptor (e.g. Diphtheria toxin receptor) in the cells allows us to specifically kill these cells upon the administration of the toxin (e.g. Diphtheria toxin). This study allows us to determine the consequence of the loss of the toxin receptor expressing cells during development and for tissue homeostasis.
If the required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models (3.4.4.7).

B. <u>No Intervention:</u>

I. No experimental interventions:

To visualize for example how stem cells behave during tissue development, we analyze in ex vivo experiments reporter mice in which e.g. stem cells are labelled with GFP.

<u>Interventions:</u>

II Experimental interventions II:

- To identify/validate which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze GM mice in which functional gene(s) will be activated, inactivated, overexpressed and/or misexpressed.
- The administration of small molecule compounds/drugs/chemicals/toxins, we might be able to rescue or mimic the phenotypes of the in vivo genetic deletion(s) and/or activation(s) and therefore further identify the function of these cells in vivo. The mice might be injected with DNA labelling agents shortly before euthanasia to measure the proliferation capacity of the stems and their derivatives.
- Injuries (such as the surgical removal of a tumor, taking biopsies (not for the analysis, but for the injury), making a wound) will be made to investigate for example the recruitment of cell types (e.g. immune cells) with subsequent effects on expression profile of different cells in the tissue and the tumor.

C. Readout parameters

To study dynamic processes that are missed in static histological images, in vivo imaging will be performed. For the imaging experiments two different strategies will be used:

1. Imaging in an acute experiment under anesthesia. This is the method of choice for the imaging of processes that need to be imaged frequently (time scale of hours) for a short period of time (max 72 hours). In this case it is not an option to anesthetize the animal

multiple times a day.
2. Chronic imaging. To study processes at a timescale of days chronic imaging is the method of choice.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

1. Tissue sampling for genotyping and identification via ear and tail biopsy resp. under anaesthesia (4% (isoflurane/oxygen).
2. Administration of transgene inducing or deleting agents or control substances alone or in combination, continuously or intermittently by one or more of the following routes:
   a) in diet or drinking water (max. 1 time, < 2 wks)
   b) subcutaneous (max. 1 time)
   c) intraperitoneal(max. 5 times)
   d) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia
   e) oral (max. 10 times)
3. Administration of small molecule compounds, drugs, toxin, chemicals or control substances alone or in combination, continuously or intermittently by one or more of the following routes:
   a) in diet or drinking water (max. time, <2 wks)
   b) subcutaneous (max. 10 time)
   c) intraperitoneal (max. 10 times)
   d) implantation of a slow release pellet subcutaneously under adequate anaesthesia and analgesia (1 time)
   e) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (1 time)
   f) oral (max. 10 times)
4. (Optional) Administration of a labelling agent (e.g. BrdU) via one of the following routes:
   a) intraperitoneal (max 3 times)
   b) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (1 time)
   c) intravenous (max 3 times)
5. (Optional) To mimic the injury resulting from e.g. surgical removal of a tumor or obtaining a biopsy, (tumor) tissues will be 'injured' under adequate anaesthesia and analgesia
6. Intravital imaging. For the imaging experiments one of the two different strategies will be used:
   1) Acute imaging: Surgical exposure of the imaging site under adequate anaesthesia and analgesia. Such an experiment will last up to 72 hours
   2) Chronic imaging: In these situation prior to the imaging period an imaging window is implanted (such as the breast, skin, abdomen and skull) (one window per animal) followed by the repeated imaging sessions under isoflurane anesthesia. From experience we know that for the implantation of windows in the skin does not lead to post-operative discomfort and post-operative analgesia is therefore extensive postoperative analgesia is not indicated. In a number of cases this is contra-indicated for the processes under study.

7) All animals will be killed while still under anesthesia for ex vivo analysis of the isolation of organs or tumors:
   a) perfusion fixation under lethal dose of Nembutal.
   b) via cervical dislocation under isoflurane anaesthesia

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.

Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments.

Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mus musculus, (Wt, genetically modified, mutants)
Origin: Hubrecht institute/external licensed breeders.
Adult: max. 5000
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand tissue development/homeostasis and cancer in the context of the whole organism. For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

For the type of experiments described in this procedure, it is difficult to calculate the exact number of animals, since for various experimental setups different amount of mice are required, and the variation and outcome of the experiments are unknown. The number of mice required depends on the mouse model (e.g tumor incidence, tumor heterogeneity), the type of intervention and the characteristics of the measuring parameters and can therefore only be described in global and strategic terms. For these reasons we have provided a total number of mice based on experience over the past 5 years with these types of experiments in our group. Before we will start an experiment we will write an application to the IVD. In this application we will exactly describe which considerations, facts and results have led to the proposition of the number of animals needed for these experiments.
We have estimated the number of animals based on experience over the past 5 years. To give an idea of typical experiments we have done in the past 5 years:

1) Acute imaging without experimental intervention. For example, we used this experiment to see how frequent Lgr5+ stem cells in the intestine divide at various locations within the stem cell niche. We know from experience that we need to image at least 12 mice to quantitatively say whether these cells have migratory potential or not. On average, we had typically 10 of this type of projects per year, so over a 5 year time span we expect to require approximately 50 of these typical experiments.
2) Acute imaging with experimental intervention. For example, we used this experiment to test the migration potential of tumor cells that have "high-jacked" stem cells properties in breast tumors, mice will be imaged acutely that overexpress PyMT oncogene in the mammary gland. We know from experience that we need to image at least 6 mice to quantitatively say whether these cells have migratory potential or not. On average, we had typically 20 of this type of projects per year, so over a 5 year time span we expect to require approximately 100 of these typical experiments.
3) Chronic imaging without experimental intervention. For example, we used this experiment to see how the progeny of Lgr5+ stem cell outcompetes the progeny of another Lgr5+ stem cell. To study this, we typically image the progeny over multiple days. We typically image at least 20 mice to quantitatively say something about competition. On average, we had typically 20 of this type of projects per year, so over a 5 year time span we expect to require approximately 100 of these typical experiments.
4) Chronic imaging with experimental intervention. For example, we used this experiment to see how the progeny of Lgr5+ stem cell in which e.g. the APC is depleted outcompetes the progeny of a wild-type Lgr5+ stem cell. To study this, we typically image the progeny over multiple days. We typically image at least 20 mice to quantitatively say something about competition. On average, we had typically 18 of this type of projects per year, so over a 5 year time span we expect to require approximately 90 of these typical experiments.

For the chronic in vivo imaging experiments in some cases the animals can no longer be used for the experiments (tumour at a location not suitable for imaging, problems with the window. This happens in <10% of the cases.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Before we consider the import or generation and subsequently analysis of (compound) GGM to study cancer models, we first will extensively analyze cell lines, existing tissue patient material and/or organoids. However, animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms that drive tissue homeostasis/development, cancer initiation, growth and metastasis.

We make extensive use of human material and in vitro experiments using cell lines and organoid culture (3D cultures) where possible, which extensively reduces the animal numbers. The use human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum.

Whenever possible, we will perform pilot studies with the minimum number of animals possible.
Where possible, mice with inducible alleles will be used, so mice should not display a phenotype before the induction of the alleles.

Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.

If homozygous mice doesn't show any discomfort we will keep the mice on a homozygous background, thereby reducing the number of mice needed for breeding.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 conditions.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

If required, isoflurane will be used general anaesthesia and Temgesic to relieve pain.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Other than in terminally anaesthetized animals dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that will result in no more than transient, light discomfort.
Due to administration of inducing agents or other substances animals will be experiencing no follow up effects.
Animals bearing tumors will never reach end-stage clinical effects. The scientific endpoints of all studies are much earlier than the humane endpoints.
It is expected that no animals (0%) will be experiencing more than mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analyzed when possible

Animals may lose their window (<5%). In the vast majority of these animals (>95%), we notice signs of detachment of the window far before it becomes lose, and the mice will be sacrificed. In the remaining animals, the window loss will be noticed within 24hrs. From prior experience we know that window loss is not a source for additional discomfort.

Explain why these effects may emerge.

Tumor development and/or inflicted tissue damage due to genetic alterations and/or administration of small molecule compounds/drugs/chemicals/toxins.
It is unclear why some animals may lose their windows.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort.
Loss of window
Growth of tumor: In all cases the guidelines of the Code of Practice Animals in Cancer research will strictly be followed.

Indicate the likely incidence.

Expected <5%, moderate <1day.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Mild discomfort : interventions resulting in mild discomfort in combination with acute imaging (15%)
Moderate discomfort: interventions resulting in moderate discomfort in combination with acute imaging (15%)
Moderate discomfort : Interventions in combination with chronic imaging (70%)

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed while still under anesthesia for imaging to use their organs in ex vivo analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80102
1.2	Provide the name of the licenced establishment.	Koninklijke Nederlandse Academie van Wetenschappen (Hubrecht Institute)

1.3 List the serial number and type of animal procedure.

Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.

Serial number	Type of animal procedure
3.4.4.3	Ex-vivo analysis in mouse models and after transplantation tissues/cells/organoids

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general design of the experiments in this appendix is shown in the flowchart in appendix 1.
Our aim is to determine in ex vivo analysis the molecular/cellular mechanisms required for tissue homeostasis/development, tumor initiation, tumor progression, cancer diagnosis, cancer prevention, and cancer treatment via the analysis of (compound) mouse models.

The different components of the proposed experiments are (see also the flowchart in appendix 1):

A. <u>Mouse models:</u>

There are several considerations to choose for a specific animal model:

- Wt mice,
- Genetically modified mice that expressed a fluorescent marker in a specific cell type/lineage. In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to induce the expression of the fluorescent marker.
- Genetically modified mice that changes expression of a functional gene(transgene knockout). In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to change the expression of the functional gene.
- Spontaneous tumor model
- Immune-deficient/wt recipient mouse

The choice will in all cases based on the combination of the following considerations:

- Aim/ specific question (normal development, tumor development, establishing cell lines/organoids or a cell type specific expression of a fluorescent marker for an

immunohistochemical study)
- Type of tumor
- Aim/readout parameters (e.g. static vs dynamic, long-term vs short-term dynamics)

B. Intervention III:

To identify/validate for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze mice in which tissues/cells/organoids are (mostly orthotopically) transplanted. The presence of e.g. a fluorescent marker in transplanted tissues/cells/organoids allows us to localize these cells by histology and to isolate the fluorescent expressing cells via FACS sorting, which allows us to analyze (gene expression profile) and culture these cells. In some cases, mice get a special diet (but this does not lead to any discomfort).

C. 1. Ex vivo analysis

In all experiments, animals will be killed adult organs will be isolated for detailed analysis of the consequences of transplantation of tissue/cells/organoids. Analysis will include among others histological sections labelled with antibodies or antisense RNA probes, RNA expression analysis, DNA or protein extracts. Also, cells from organs might be isolated by FACS and/or cultured in vitro (organoids).

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

1. Tissue sampling for genotyping and identification via ear and tail biopsy resp. under anaesthesia (4% (isoflurane/oxygen).
2. Transplantation of tissues/cells/organoids under adequate anaesthesia and analgesia
3. Administration of transgene inducing or deleting agents or control substances alone or in combination, continuously or intermittently by one or more of the following routes:

a) in diet or drinking water (max. 1 time, < 2 wks)
b) subcutaneous (max. 1 time)
c) intraperitoneal(max. 5 times)
d) implantation of a slow release pellet subcutaneously under adequate anaesthesia and analgesia (1 time)
e) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (1 time)
f) oral (max. 10 times)

4. (Optional) Administration of a labelling agent (e.g. BrdU) via one of the following routes:

a) intraperitoneal (max 3 times)
b) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (1 time)
c) intravenous (max 3 times)

5. All animals will be killed and organ(s)/tissue will be isolated for ex vivo analysis

Adult mice: via CO2/O2 method or perfusion fixation under lethal dose of Nembutal.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mus musculus, (Wt, genetically modified, mutants)
Origin: Hubrecht institute/external licensed breeders.
Adult: max. 4500
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand tissue development/homeostasis and cancer in the context of the whole organism. For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

For the type of experiments described in this procedure, it is difficult to calculate the exact number of animals, since for various experimental setups different amount of mice are required, and the variation and outcome of the experiments are unknown. The number of mice required depends on the mouse model (e.g tumor incidence, tumor heterogeneity), the type of intervention and the characteristics of the measuring parameters and can therefore only be described in global and strategic terms. For these reasons we have provided a total number of mice based on experience over the past 5 years with these types of experiments in our group. Before we will start an experiment we will write an application to the IVD. In this application we will exactly describe which considerations, facts and results have led to the proposition of the number of animals needed for these experiments.
To give an idea of a typical experiment in this appendix: E.g. if we want to characterize tumor cells that have "high-jacked" stem cell properties in colorectal tumors, these cells need to be isolated by flow cytometry to subsequently analyze them by RNAseq. For example, for this experiment we want to isolate pieces of colon from mice in which the tumor suppressor gene APC is depleted in the stem cells (via lgr5-GFP ires-creert). These mice develop tumors throughout the intestine, and reach human end point, due to dysfunctional intestine, before tumors progress to a stage in which cells acquire stem cell properties. Therefore, pieces of colon are transplanted in recipient mice to initiate the growth of a single tumor. Since just one tumor is formed upon transplantation, the intestine is functional and the tumor can progress to a stage where cells adapt stem cell properties and start to express GFP by the Lgr5 stem cell promotor. Based on the expression of GFP, these cells can then be isolated by flow cytometry. For such a typical experiment, a maximum of 30 mice may be required (15 donor mice and 15 recipient mice per group). For some experiments, multiple time points are required. On average, we had typically 30 of this type of experiments per year, so over a 5 year time span we expect to require approximately 150 of these typical experiments.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Before we consider the import or generation and subsequently analysis of (compound) GGM to study cancer models, we first will extensively analyze cell lines, existing tissue patient material and/or organoids. However, animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms that drive tissue homeostasis/development, cancer initiation, growth and metastasis.

We make extensive use of human material and in vitro experiments using cell lines and organoid culture (3D cultures) where possible, which extensively reduces the animal numbers. The use human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum.

Whenever possible, we will perform pilot studies with the minimum number of animals possible.

Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 conditions.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

If required, isoflurane will be used general anaesthesia and Temgesic to relieve pain.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Other than in terminally anaesthetized animals dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that will result in no more than transient, light discomfort. In less than 3% of all experiments, pumps/pellets will be implanted which leads to a moderate discomfort.
Due to administration of gene-inducing agents animals will be experiencing no follow up effects.
Animals bearing tumors will never reach end-stage clinical effects. The scientific endpoints of all studies are much earlier then the humane endpoints.
It is expected that no animals (0%) will be experiencing more then mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day.

Explain why these effects may emerge.

Tumordevelopment and/or inflicted tissue damage due to genetic alterations

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort.
In all cases the guidelines of the Code of Practice Animals in Cancer research will strictly be followed

Indicate the likely incidence.

Expected 0% within time frame of the experiments.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Adult: mild 96%, moderate 1% <1day, moderate <3% constantly due to implantation pellet/pump

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed to use their organs in ex vivo analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80102
1.2	Provide the name of the licenced establishment.	Koninklijke Nederlandse Academie van Wetenschappen (Hubrecht Institute)

1.3 List the serial number and type of animal procedure.

Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.

Serial number	Type of animal procedure
3.4.4.4	In vivo imaging in mouse models and after transplantation tissues/cells/organoids

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general design of the experiments in this appendix is shown in the attached flowchart.

Our aim is to determine with in vivo imaging the molecular/cellular mechanisms required for tissue homeostasis/development, tumor initiation, tumor progression, cancer diagnosis, cancer prevention, and cancer treatment via the analysis of (compound) mouse models.

The different components of the proposed experiments are (see also the flowchart in appendix 1):

A. <u>Mouse models:</u>

There are several considerations to choose for a specific animal model:

- Wt mice,
- Genetically modified mice that expressed a fluorescent marker in a specific cell type/lineage. In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to induce the expression of the fluorescent marker.
- Genetically modified mice that changes expression of a functional gene(transgene knockout). In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to change the expression of the functional gene.
- Spontaneous tumor model
- Immune-deficient/wt recipient mouse

The choice will in all cases based on the combination of the following considerations:

- Aim/ specific question (normal development, tumor development, establishing cell

lines/organoids or a cell type specific expression of a fluorescent marker)
- Type of tumor
- Aim/readout parameters (e.g. static vs dynamic, long-term vs short-term dynamics)

B. Intervention III:

To identify/validate for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze mice in which tissues/cells/organoids are (mostly orthotopically) transplanted. The presence of e.g. a fluorescent marker in transplanted tissues/cells/organoids allows us to visualize these cells by intravital imaging. In some cases, mice get a special diet (but this does not lead to any discomfort).

C. Readout parameters

To study dynamic processes that are missed in static histological images, in vivo imaging will be performed. For the imaging experiments two different strategies will be used:

2. Imaging in an acute experiment under anaesthesia. This is the method of choice for the imaging of processes that need to be imaged frequently (time scale of hours) for a short period of time (max 72 hours). In this case it is not an option to anesthetize the animal multiple times a day.

Chronic imaging. To study processes at a timescale of days chronic imaging is the method of choice.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

1. Tissue sampling for genotyping and identification via ear and tail biopsy resp. under anaesthesia (4% (isoflurane/oxygen).
2. Transplantation of tissues/cells/organoids under adequate anaesthesia and analgesia
3. Intravital imaging either:
   - Acute imaging: Surgical exposure of the imaging site under adequate anaesthesia and analgesia. Such an experiment will last up to 72 hours
   - Chronic imaging: In these situation prior to the imaging period an imaging window is implanted (such as the breast, skin, abdomen and skull) (one window per animal) followed by the repeated imaging sessions under isoflurane anesthesia. From experience we know that for the implantation of intracutaneous windows does not lead to post-operative discomfort and post-operative analgesia is therefore extensive postoperative analgesia is not indicated. In a number of cases this is contraindicated for the processes under study.
4. All animals will be killed while still under anesthesia for ex vivo analysis of the isolation of organs or tumors:
   a) perfusion fixation under lethal dose of Nembutal.
   b) via cervical dislocation under isoflurane anesthesia

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments.
Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mus musculus, (Wt, genetically modified, mutants)
Origin: Hubrecht institute/external licensed breeders.
Adult: max. 2000
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand tissue development/homeostasis and cancer in the context of the whole organism. For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

For the type of experiments described in this procedure, it is difficult to calculate the exact number of animals, since for various experimental setups different amount of mice are required, and the variation and outcome of the experiments are unknown. The number of mice required depends on the mouse model (e.g tumor incidence, tumor heterogeneity), the type of intervention and the characteristics of the measuring parameters and can therefore only be described in global and strategic terms. For these reasons we have provided a total number of mice based on experience over the past 5 years with these types of experiments in our group. Before we will start an experiment we will write an application to the IVD. In this application we will exactly describe which considerations, facts and results have led to the proposition of the number of animals needed for these experiments.
To give an idea of a typical experiment in this appendix: E.g. if we want to characterize whether the tumor cells that have "high-jacked" stem cell properties have also acquired migratory properties, the motility of these cells need to be visualized by intravital microscopy. For example, for this experiment we want to isolate pieces of colon from mice in which the tumor suppressor gene APC is depleted in the stem cells (via lgr5-GFP ires-creert). These mice develop tumors throughout the intestine, and reach human end point, due to dysfunctional intestine, before tumors progress to a stage in which cells acquire stem cell properties. Therefore, pieces of colon are transplanted in recipient mice to initiate the growth of a single tumor. Since just one tumor is formed upon transplantation, the intestine is functional and the tumor can progress to a stage where cells adapt stem cell properties and start to express GFP by the Lgr5 stem cell promotor. These cells can then be visualized by acute intravital imaging to visualize e.g. cell migration and chronic intravital imaging to visualize e.g. the formation of progeny. For such a typical experiment, a maximum of 20 mice may be required (10 donor mice and 10 recipient mice per group).

1) Acute imaging: On average, we had typically 4 of this type of projects per year, so over a 5 year time span we expect to require approximately 20 of these typical experiments.
2) Chronic imaging: On average, we had typically 16 of this type of projects per year, so over a 5 year time span we expect to require approximately 80 of these typical experiments.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Before we consider the import or generation and subsequently analysis of (compound) GGM to study cancer models, we first will extensively analyze cell lines, existing tissue patient material and/or organoids. However, animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms that drive tissue homeostasis/development, cancer initiation, growth

and metastasis.

We make extensive use of human material and in vitro experiments using cell lines and organoid culture (3D cultures) where possible, which extensively reduces the animal numbers. The use human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum.

Whenever possible, we will perform pilot studies with the minimum number of animals possible.

Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 conditions

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken

to ensure that optimal procedures are used.

If required, isoflurane will be used general anaesthesia and Temgesic to relieve pain.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Other than in terminally anaesthetized animals dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that will result in no more than transient, light discomfort.
Due to administration of inducing agents or other substances animals will be experiencing no follow up effects.
Animals bearing tumors will never reach end-stage clinical effects. The scientific endpoints of all studies aremuch earlier then the humane endpoints.
It is expected that no animals (0%) will be experiencing more then mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analyzed when possible.

Animals may lose their window (<5%). In the vast majority of these animals (>95%), we notice signs of detachment of the window far before it becomes lose, and the mice will be sacrificed. In the remaining animals, the window loss will be noticed within 24hrs. From prior experience we know that window loss is not a source for additional discomfort.

Explain why these effects may emerge.

Tumordevelopment and/or inflicted tissue damage due to transplantation of tissues/cells/organoids.
It is unclear why some animals may lose their windows.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort.
Loss of window
Growth of tumor: In all cases the guidelines of the Code of Practice Animals in Cancer research will strictly be followed.

Indicate the likely incidence.

Expected <5%, moderate <1day.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Mild discomfort : mild discomfort due to intervention, acute imaging (12%)
Moderate discomfort: moderate discomfort due to intervention, acute imaging (8%)
Moderate discomfort : All chronic imaging experiments (80%)

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed while still under anesthesia for imaging to use their organs in ex vivo analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80102
1.2	Provide the name of the licenced establishment.	Koninklijke Nederlandse Academie van Wetenschappen (Hubrecht Institute)

1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number	Type of animal procedure
		3.4.4.5	Ex-vivo analysis in mouse models and after transplantation tissues/cells/organoids, Gene inactivation/overexpression, administering drugs and injury

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general design of the experiments in this appendix is shown in the attached flowchart.
Our aim is to determine in ex vivo analysis the molecular/cellular mechanisms required for tissue homeostasis/development, tumor initiation, tumor progression, cancer diagnosis, cancer prevention, and cancer treatment via the analysis of (compound) mouse models.

The different components of the proposed experiments are (see also the flowchart in appendix 1):

A. <u>Mouse models:</u>

There are several considerations to choose for a specific animal model:

- Wt mice,
- Genetically modified mice that expressed a fluorescent marker in a specific cell type/lineage. In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to induce the expression of the fluorescent marker.
- Genetically modified mice that changes expression of a functional gene(transgene knockout). In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to change the expression of the functional gene.
- Spontaneous tumor model
- Immune-deficient/wt recipient mouse

The choice will in all cases based on the combination of the following considerations:

- Aim/ specific question (normal development, tumor development, establishing cell lines/organoids or a cell type specific expression of a fluorescent marker for an immunohistochemical study)
- Type of tumor
- Aim/readout parameters (e.g. static vs dynamic, long-term vs short-term dynamics)

To identify/validate for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze (GM) mice in which cells/tissues/organoids are transplanted and the gene(s) will be activated, inactivated, overexpressed and/or misexpressed. In some cases, mice get a special diet (but this does not lead to any discomfort).
We will use inducible systems including e.g. the well-known Cre-LoxP system in which a floxed gene can be deleted, overexpressed or misexpressed upon the induction of the Cre enzyme via the administration of tamoxifen. In particular, when Cre is expressed in mice harboring a LoxP-containing target gene, the desired gene modification can be restricted to certain developmental stage, organ (e.g. intestine), cell type (e.g. stem cell) of the mouse depending on the specificity and timing of recombinase expression. In this way the desired gene modification can be restricted to certain developmental stage, organ (e.g. intestine), cell type (e.g. stem cell). Successful deletion/activation via (e.g.) Cre enzyme induction might be monitored via expression of a reporter gene (e.g. LacZ or fluorescent protein(s)).

We will also use endogenous and exogenous promotors that are tissue or cell specific to drive expression of genes.
The presence of e.g. a fluorescent marker in a (putative) stem cell allows us to localize these cells by histology and to isolate the fluorescent expressing cells via FACS sorting, which allows us to analyze (gene expression profile) and culture these cells.
The introduction of e.g. a toxin receptor (e.g., Diphtheria toxin receptor) in the cells allows us to specifically kill these cells upon the administration of the toxin (e.g., Diphtheria toxin). This study allows us to determine the consequence of the loss of the toxin receptor expressing cells during development and for tissue homeostasis.
If the required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models (3.4.4.7).

B. <u>Interventions IV:</u>

- To identify/validate for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze mice in which tissues/cells/organoids are (mostly orthotopically) transplanted. The presence of e.g. a fluorescent marker in transplanted tissues/cells/organoids allows us to visualize these cells by intravital imaging.To identify/validate which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze GM mice in which functional gene(s) will be activated, inactivated, overexpressed and/or misexpressed.
- The administration of small molecule compounds/drugs/chemicals/toxins, we might be able to rescue or mimic the phenotypes of the in vivo genetic deletion(s) and/or activation(s) and therefore further identify the function of these cells in vivo.The mice might be injected with DNA labelling agents shortly before euthanasia to measure the proliferation capacity of the stems and their derivatives.
- Injuries (such as the surgical removal of a tumor, taking biopsies (not for the analysis, but for the injury), making a wound) will be made to investigate for example the recruitment of cell types (e.g. immune cells) with subsequent effects on expression profile of different cells in the tissue and the tumor.

C. Ex vivo analysis

In all experiments, animals will be killed adult organs will be isolated for detailed analysis of the consequences of transplantation of tissue/cells/organoids. Analysis will include among others histological sections labelled with antibodies or antisense RNA probes, RNA expression analysis, DNA or protein extracts. Also, cells from organs might be isolated by FACS and/or cultured in vitro (organoids).

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

1. Tissue sampling for genotyping and identification via ear and tail biopsy resp. under anaesthesia (4% (isoflurane/oxygen).
2. Transplantation of tissues/cells/organoids under adequate anaesthesia and analgesia
3. Administration of transgene inducing or deleting agents or control substances alone or in combination, continuously or intermittently by one or more of the following routes:
   a) in diet or drinking water (max. 1 time, < 2 wks)
   b) subcutaneous (max. 3 time)
   c) intraperitoneal(max. 7 times)
   d) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia
   e) oral (max. 10 times)
4. Administration of small molecule compounds, drugs, toxin, chemicals or control substances alone or in combination, continuously or intermittently by one or more of the following routes:
   a) in diet or drinking water (max. time, <2 wks)
   b) subcutaneous (max. 10 time)
   c) intraperitoneal (max. 10 times)
   d) implantation of a slow release pellet subcutaneously under adequate anaesthesia and analgesia (1 time)
   e) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (1 time)
   f) oral (max. 10 times)
5. (Optional) Administration of a labelling agent (e.g. BrdU) via one of the following routes:
   a) intraperitoneal (max 3 times)
   b) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (1 time)
   c) intravenous (max 3 times)
6. (Optional) To mimic the injury resulting from e.g. surgical removal of a tumor or obtaining a biopsy, (tumor) tissues will be 'injured' under adequate anesthesia and analgesia
7. All animals will be killed and organ(s)/tissue will be isolated for ex vivo analysis
   a) Adult mice: via CO2/O2 method or perfusion fixation under lethal dose of Nembutal.
   b) Embryo's and neonates: will be put on melting ice water for 10 min. (but not in contact with) after which they will be decapitated and the head immediately frozen (or the brains dissected and fixed).

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mus musculus, (Wt, genetically modified, mutants)
Origin: Hubrecht institute/external licensed breeders.
Adult: max. 1650
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand tissue development/homeostasis and cancer in the context of the whole organism. For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

For the type of experiments described in this procedure, it is difficult to calculate the exact number of animals, since for various experimental setups different amount of mice are required, and the variation and outcome of the experiments are unknown. The number of mice required depends on the mouse model (e.g tumor incidence, tumor heterogeneity), the type of intervention and the characteristics of the measuring parameters and can therefore only be described in global and strategic terms. For these reasons we have provided a total number of mice based on experience over the past 5 years with these types of experiments in our group. Before we will start an experiment we will write an application to the IVD. In this application we will exactly describe which considerations, facts and results have led to the proposition of the number of animals needed for these experiments.
To give an idea of a typical experiment in this appendix: E.g. if we want to characterize how inactivation of a gene (e.g. Kras) affects tumor cells that have "high-jacked" stem cell properties in colorectal tumors, these cells need to be isolated by flow cytometry to subsequently analyze them by RNAseq. For example, for this experiment we want to isolate pieces of colon from mice in which the tumor suppressor gene APC and Kras are depleted in the stem cells (via lgr5-GFP ires-CreERTt). These mice develop tumors throughout the intestine, and reach human end point, due to dysfunctional intestine, before tumors progress to a stage in which cells acquire stem cell properties. Therefore, pieces of colon are transplanted in recipient mice to initiate the growth of a single tumor. Since just one tumor is formed upon transplantation, the intestine is functional and the tumor can progress to a stage where cells adapt stem cell properties and start to express GFP by the Lgr5 stem cell promotor. Based on the expression of GFP, these cells can then be isolated by flow cytometry. For such a typical experiment, a maximum of 60 mice may be required (15 donor mice and 15 recipient mice per group. We need two groups: one control group in which Kras is not depleted and one group in which Kras is depleted). On average, we had typically 5 to 6 of this type of projects per year, so over a 5 year time span we expect to require approximately 27,5 of these typical experiments.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Before we consider the import or generation and subsequently analysis of (compound) GGM to study cancer models, we first will extensively analyze cell lines, existing tissue patient material and/or organoids. However, animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms that drive tissue homeostasis/development, cancer initiation, growth and metastasis.

We make extensive use of human material and in vitro experiments using cell lines and organoid culture (3D cultures) where possible, which extensively reduces the animal numbers. The use human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum.

Whenever possible, we will perform pilot studies with the minimum number of animals possible.
Where possible, mice with inducible alleles will be used, so mice should not display a phenotype before the induction of the alleles.

Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.

If homozygous mice doesn't show any discomfort we will keep the mice on a homozygous background, thereby reducing the number of mice.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 conditions.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

If required, isoflurane will be used as dormicum and Temgesic to releave pain.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Other than in terminally anaesthetized animals dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that will result in no more than transient, light discomfort. In less than 3% of all experiments, pumps/pellets will be implanted which leads to a moderate discomfort.
Due to administration of inducing agents or other substances animals will be experiencing no follow up effects.
Animals bearing tumors will never reach end-stage clinical effects. The scientific endpoints of all studies are much earlier then the humane endpoints.
It is expected that no animals (0%) will be experiencing more then mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analyzed when possible.

Explain why these effects may emerge.

Tumor development and/or inflicted tissue damage due to genetic alterations and/or administration of small molecule compounds/drugs/chemicals/toxins.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort.
In all cases the guidelines of the Code of Practice Animals in Cancer research will strictly be followed

Indicate the likely incidence.

Expected 0% within time frame of the experiments.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Adult: mild 96%, moderate 1% <1day, moderate <3% constantly due to implantation pellet/pump

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed to use their organs in ex vivo analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.
☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80102
1.2	Provide the name of the licenced establishment.	Koninklijke Nederlandse Academie van Wetenschappen (Hubrecht Institute)

1.3 List the serial number and type of animal procedure.

Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.

Serial number	Type of animal procedure
3.4.4.6	In vivo imaging in mouse models and after transplantation tissues/cells/organoids, Gene inactivation/overexpression, administering drugs and injury

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general design of the experiments in this appendix is shown in the attached flowchart.
Our aim is to determine in ex vivo analysis the molecular/cellular mechanisms required for tissue homeostasis/development, tumor initiation, tumor progression, cancer diagnosis, cancer prevention, and cancer treatment via the analysis of (compound) mouse models.

The different components of the proposed experiments are (see also the flowchart in appendix 1):

A. Mouse models:

There are several considerations to choose for a specific animal model:

- Wt mice,
- Genetically modified mice that expressed a fluorescent marker in a specific cell type/lineage. In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to induce the expression of the fluorescent marker.
- Genetically modified mice that changes expression of a functional gene(transgene knockout). In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to change the expression of the functional gene.
- Spontaneous tumor model
- Immune-deficient/wt recipient mouse

The choice will in all cases based on the combination of the following considerations:

- Aim/ specific question (normal development, tumor development, establishing cell lines/organoids or a cell type specific expression of a fluorescent marker for an immunohistochemical study)
- Type of tumor
- Aim/readout parameters (e.g. static vs dynamic, long-term vs short-term dynamics)

To identify/validate for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze (GM) mice in which cells/tissues/organoids are transplanted and the gene(s) will be activated, inactivated, overexpressed and/or misexpressed. In some cases, mice get a special diet (but this does not lead to any discomfort).
We will use inducible systems including e.g. the well-known Cre-LoxP system in which a floxed gene can be deleted, overexpressed or misexpressed upon the induction of the Cre enzyme via the administration of tamoxifen. In particular, when Cre is expressed in mice harboring a LoxP-containing target gene, the desired gene modification can be restricted to certain developmental stage, organ (e.g. intestine), cell type (e.g. stem cell) of the mouse depending on the specificity and timing of recombinase expression. In this way the desired gene modification can be restricted to certain developmental stage, organ (e.g. intestine), cell type (e.g. stem cell). Successful deletion/activation via (e.g.) Cre enzyme induction might be monitored via expression of a reporter gene (e.g. LacZ or fluorescent protein(s)).

We will also use endogenous and exogenous promotors that are tissue or cell specific to drive expression of genes.
The presence of e.g. a fluorescent marker in a (putative) stem cell allows us to localize these cells by in vivo imaging.
The introduction of e.g. a toxin receptor (e.g., Diphtheria toxin receptor) in the cells allows us to specifically kill these cells upon the administration of the toxin (e.g., Diphtheria toxin). This study allows us to determine the consequence of the loss of the toxin receptor expressing cells during development and for tissue homeostasis.
If the required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models (3.4.4.7).

B. <u>Interventions IV:</u>

- To identify/validate for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze mice in which tissues/cells/organoids are (mostly orthotopically) transplanted. The presence of e.g. a fluorescent marker in transplanted tissues/cells/organoids allows us to visualize these cells by intravital imaging. To identify/validate which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze GM mice in which functional gene(s) will be activated, inactivated, overexpressed and/or misexpressed.
- The administration of small molecule compounds/drugs/chemicals/toxins, we might be able to rescue or mimic the phenotypes of the in vivo genetic deletion(s) and/or activation(s) and therefore further identify the function of these cells in vivo. The mice might be injected with DNA labelling agents shortly before euthanasia to measure the proliferation capacity of the stems and their derivatives.
- Injuries (such as the surgical removal of a tumor, taking biopsies (not for the analysis, but for the injury), making a wound) will be made to investigate for example the recruitment of cell types (e.g. immune cells) with subsequent effects on expression profile of different cells in the tissue and the tumor.

C. Readout parameters

To study dynamic processes that are missed in static histological images, in vivo imaging will be performed. For the imaging experiments one of the two different strategies will be used:

1. Imaging in an acute experiment under anesthesia. This is the method of choice for the imaging of processes that need to be imaged frequently (time scale of hours) for a short period of time (max 72 hours). In this case it is not an option to anesthetize the animal multiple times a day.
2. Chronic imaging. To study processes at a timescale of days chronic imaging is the method of choice.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

1. Tissue sampling for genotyping and identification via ear and tail biopsy resp. under anaesthesia (4% (isoflurane/oxygen).
2. Transplantation of tissues/cells/organoids under adequate anaesthesia and analgesia
3. Administration of transgene inducing or deleting agents or control substances alone or in combination, continuously or intermittently by one or more of the following routes:
   a) in diet or drinking water (max. 1 time, < 2 wks)
   b) subcutaneous (max. 3 time)
   c) intraperitoneal(max. 7 times)
   d) implantation of a slow release pellet subcutaneously under adequate anesthesia and analgesia (1 time)
   e) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (1 time)
   f) oral (max. 10 times)
4. Administration of small molecule compounds, drugs, toxin, chemicals or control substances alone or in combination, continuously or intermittently by one or more of the following routes:
   a) in diet or drinking water (max. time, <2 wks)
   b) subcutaneous (max. 10 time)
   c) intraperitoneal (max. 10 times)
   d) implantation of a slow release pellet subcutaneously under adequate anaesthesia and analgesia (1 time)
   e) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (1 time)
   f) oral (max. 10 times)
5. (Optional) Administration of a labelling agent (e.g. BrdU) via one of the following routes:
   a) intraperitoneal (1 time)
   b) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (1 time)
   c) intravenous (1 time)
6. (Optional) To mimic the injury resulting from e.g. surgical removal of a tumor or obtaining a biopsy, (tumor) tissues will be 'injured' under adequate anaesthesia and analgesia
7. Intravital imaging either

   - Acute imaging: Surgical exposure of the imaging site under adequate anaesthesia and analgesia. Such an experiment will last up to 72 hours
   - Chronic imaging: In these situation prior to the imaging period an imaging window is implanted (such as the breast, skin, abdomen and skull) (one window per animal) followed by the repeated imaging sessions under isoflurane anesthesia. From experience we know that for the implantation of intracutaneous windows does not lead to post-operative discomfort and post-operative analgesia is therefore extensive postoperative analgesia is not indicated. In a number of cases this is contraindicated for the processes under study.
8. All animals will be killed and organ(s)/tissue will be isolated for ex vivo analysis
   a) Adult mice: via CO2/O2 method or perfusion fixation under lethal dose of Nembutal.
   b) Embryo's and neonates: will be put on melting ice water for 10 min. (but not in contact with) after which they will be decapitated and the head immediately frozen (or the brains dissected and fixed).

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mus musculus, (Wt, genetically modified, mutants)
Origin: Hubrecht institute/external licensed breeders.
Adult: max. 2800
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand tissue development/homeostasis and cancer in the context of the whole organism. For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

For the type of experiments described in this procedure, it is difficult to calculate the exact number of animals, since for various experimental setups different amount of mice are required, and the variation and outcome of the experiments are unknown. The number of mice required depends on the mouse model (e.g tumor incidence, tumor heterogeneity), the type of intervention and the characteristics of the measuring parameters and can therefore only be described in global and strategic terms. For these reasons we have provided a total number of mice based on experience over the past 5 years with these types of experiments in our group. Before we will start an experiment we will write an application to the IVD. In this application we will exactly describe which considerations, facts and results have led to the proposition of the number of animals needed for these experiments.
To give an idea of a typical experiment in this appendix: E.g. if we want to characterize how inactivation of a gene (e.g. Kras) affects the migration properties of tumor cells that have "high-jacked" stem cell properties, the motility of these cells need to be visualized by intravital microscopy. For example, for this experiment we want to isolate pieces of colon from mice in which the tumor suppressor gene APC and Kras are depleted in the stem cells (via lgr5-GFP ires-creert). These mice develop tumors throughout the intestine, and reach human end point, due to dysfunctional intestine, before tumors progress to a stage in which cells acquire stem cell properties. Therefore, pieces of colon are transplanted in recipient mice to initiate the growth of a single tumor. Since just one tumor is formed upon transplantation, the intestine is functional and the tumor can progress to a stage where cells adapt stem cell properties and start to express GFP by the Lgr5 stem cell promotor. Based on the expression of GFP, stem cells can be visualized by acute intravital imaging to visualize e.g. cell migration and chronic intravital imaging to visualize e.g. the formation of progeny. For such a typical experiment, a maximum of 40 mice may be required (10 donor mice and 10 recipient mice per group. We need two groups: one control group in which Kras is not depleted and one group in which Kras is depleted).

1) Acute imaging: On average, we had typically 9 of this type of projects per year, so over a 5 year time span we expect to require approximately 45 of these typical experiments.
2) Chronic imaging: On average, we had typically 5 of this type of projects per year, so over a 5 year time span we expect to require approximately 25 of these typical experiments.

For the chronic in vivo imaging experiments in some cases the animals can no longer be used for the experiments (tumour at a location not suitable for imaging, problems with the window. This happens in 10%< of the cases.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Before we consider the import or generation and subsequently analysis of (compound) GGM to study cancer models, we first will extensively analyze cell lines, existing tissue patient material and/or organoids. However, animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms that drive tissue homeostasis/development, cancer initiation, growth and metastasis.

We make extensive use of human material and in vitro experiments using cell lines and organoid culture (3D cultures) where possible, which extensively reduces the animal numbers. The use human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum.

Whenever possible, we will perform pilot studies with the minimum number of animals possible.
Where possible, mice with inducible alleles will be used, so mice should not display a phenotype before the induction of the alleles.

Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps..

If homozygous mice doesn't show any discomfort we will keep the mice on a homozygous background, thereby reducing the number of mice.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 conditions.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

If required, isoflurane will be used general anaesthesia and Temgesic to relieve pain.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Other than in terminally anaesthetized animals dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that will result in no more than transient, light discomfort. In less than 3% of all experiments, pumps/pellets will be implanted which leads to a moderate discomfort.
Due to administration of inducing agents or other substances animals will be experiencing no follow up effects.
Animals bearing tumors will never reach end-stage clinical effects. The scientific endpoints of all studies are much earlier then the humane endpoints.
It is expected that no animals (0%) will be experiencing more then mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analyzed when possible.

Animals may lose their window (<5%). In the vast majority of these animals (>95%), we notice signs of detachment of the window far before it becomes lose, and the mice will be sacrificed. In the remaining animals, the window loss will be noticed within 24hrs. From prior experience we know that window loss is not a source for additional discomfort.

Explain why these effects may emerge.

Tumor development and/or inflicted tissue damage due to genetic alterations and/or administration of small molecule compounds/drugs/chemicals/toxins.

It is unclear why some animals may lose their windows.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort.
Loss of window
Growth of tumor: In all cases the guidelines of the Code of Practice Animals in Cancer research will strictly be followed.

Indicate the likely incidence.

Expected <5%, moderate <1day.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Moderate discomfort: 100%

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed to use their organs in ex vivo analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80102
1.2	Provide the name of the licenced establishment.	Koninklijke Nederlandse Academie van Wetenschappen (Hubrecht Institute)

1.3 List the serial number and type of animal procedure.

Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.

Serial number	Type of animal procedure
3.4.4.7	Generation, welfare assessment and breeding of GMM/mutants with hampered phenotype

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

Creation of genetically mice via DNA/RNA injection into oocyte, injection of genetically modified ES cells into blastocysts and/or via the CRISPR/Cas9 system.

Welfare assessment according to the Consensus document on genetically altered animals . Newcompound mouse models and new created transgenic lines and/or KO lines generated via classical methods and/or novel combinations of these aforementioned lines will be monitored for 2 generations to determine the absence or presence a phenotype with constitutional discomfort.

For some transplantation experiments (e.g. when human organoids are transplanted), immune deficient acceptor mice are required. We breed our own immune deficient NOD-SCID mice. For this, we have 6-10 breeding pairs that will be replaced every 6 month that leads to offspring that will be used for experiments. Since these mice are housed under proper barrier conditions, they do not have a hampered phenotype. According to the Consensus document on genetically altered animals, breeding with these animals is considered an animal experiment and should therefore be part of the license.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Generation of new lines:
1) Superovulation.
a) Administration of gonadotropin's (2 times) by subcutaneous or intraperitoneal injections followed by mating.
b) Animals will be killed for the isolation of early embryos.

2) Embryo recipients.
a) Recipients for embryo transfer will be rendered pseudo-pregnant by mating with a sterile (vasectomized) male.
b) Genetically modified embryos will be implanted surgically or non-surgically into the reproductive tract.
c) Embryo recipients, not as part of an experiment, will be killed after weaning of the pups at three weeks of age.

3) Weaned pups at 3 weeks of age: Tissue sampling for genotyping and/or identification via tail and ear cut, respectively, under anesthesia (isoflurane).

Animals are killed by O2/CO2 method.

Breeding immune deficient acceptor mice.

Welfare assessment:
We daily check the mice on several parameters (overall appearance, size, confirmation and growth, coat condition, behavior, clinical signs, relative size and numbers) as has been described in the Directive 2010/63/EU: corrigendum of 24 Jan. 2013.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Statistical analysis doesn't play a role for these types of experiments. We will use state of the art techniques. All techniques are proven to be effective in generating GM mice with a minimum number of mice possible.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mus musculus: genetically modified and wild type adult mice. All vasectomized males which will be obtained from a registered commercial company, all other mice are obtained from our own Institute, an establishment licensed breeder by the NVWA, or from a registered commercial company.

Generation of GG mice: we expect to generate max. 10 new lines over the next 5 years. For the creation of a new GM mouse line we will use on average max. 150 mice (according to the besluit biotechnologie). Therefore in total max. 1.500 mice.

Welfare assessment: we expect to generate over the next 5 years 10 new (compound) GM lines for which we have to perform the welfare assessment. For 2 generations, 7 males and 7 females control and GM mice. We therefore need in total: 10 (new (compound) lines) * 2 (generation) * 28 ((7 male +7 female = 14 GM mice) + (7 male + 7 female = 14 control mice))= 560 mice.

Therefore in total max. 1.500 (generation of GGM) + 560 (welfare assessment) = 2060 mice
Of note
The majority of the newly generated GG mice will be floxed mice which are not part of the welfare assessment protocol.
We will not breed mice showing a hampered phenotype and but instead will sacrifice them.

For some transplantation experiments (e.g. when human organoids are transplanted), immune

deficient acceptor mice are required. We breed our own immune deficient mice. For this, we have 6-10 breeding pairs that will be replaced every 6 month that leads to offspring that will be used for experiments. So the max number of animals will be: 10 (pairs) x 2 (male and female) x 2 (twice a year) x 5 (for 5 years) = 200 animals for breeding.

These 10 breeding pairs generate on average 28 pups per week that will be used in appendix 3.4.4.3, 3.4.4.4, 3.4.4.5 and 3.4.4.6. These animals are described and counted in these appendixes.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Before we consider the generation of a new (compound) GM Mice we first will extensively analyze cell lines, existing tissue patient material and/or organoids. Only the in vitro experiments do not provide sufficient information or does not address completely the research question/hypothesis, we will consider the generation of a novel GM mice.
Animal studies are unavoidable if we seek comprehensive knowledge and understanding of molecular and cellular mechanisms of tissue homeostasis and cancer.
The CRISPR/Cas9 system allows us, if required, to genetically modify up to 5 different genes at the same time. This strongly reduce the number of mice used for the generation and/or breeding of these compound mice.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 conditions

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

If required, isoflurane will be used general anaesthesia and Temgesic to relieve pain.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

We don´t expect to find other adverse effect. This is the direct result of how we create our constructs for the generation of GM mice

Explain why these effects may emerge.

We don´t expect to find other adverse effect

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☒ No > Continue with question K.

☐ Yes > Describe the criteria that will be used to identify the humane endpoints.

Indicate the likely incidence.

Expected <5%, moderate <1day.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Donors: moderate 100%
Fosters: moderate 100%
GM mice: no 99%
GM mice: mild 1%
Immune deficient mice: no 100%
Monitoring mice: no 100%

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed to use their organs in ex vivo analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

AVD-801002015125 Overzicht aantal muizen, groepen en ongerief

Totaal aantallen: embryo's 200, pasgeboren 100 en volwassen 22.210
Muizen: 52% mild ongerief en 48% matig ongerief

	Procedure	Group	Animals	mild	moderate	Total group
3.4.4.1	Gene (in) activation interventions (ex vivo analysis)	Group 1a	Mice embryo's (>E13)	200		
		Group 1b	Mice neonates (until weaning)	100		
		Group 1c	Mice adults 99%	3840		
		Group 1d	Mice adults 4%		160	4300
3.4.4.2	Gene (in) activation interventions (in vivo imaging)	Group 2a	Mice adults 15%	750		
		Group 2b	Mice adults 85%		4250	5000
3.4.4.3	Transplantation (ex vivo analysis)	Group 3a	Mice adults 96%	4320		
		Group 3b	Mice adults 4%		180	4500
3.4.4.4	Transplantation (in vivo imaging)	Group 4a	Mice adults 12%	240		
		Group 4b	Mice adults 88%		1760	2000
3.4.4.5	Transplantation Gene (in) activation interventions (ex vivo analysis)	Group 5a	Mice adults 96%	1584		
		Group 5b	Mice adults 4%		66	1650
3.4.4.6	Transplantation Gene (in) activation interventions (in vivo imaging)	Group 6	Mice adults 20%		2800	2800
3.4.4.7	Generation Welfare assessment and Breeding of GMM	Group 7a	Mice adults 100% (generation GMM)		1500	
		Group 7b	Mice adults (welfare assessment)	554		
		Group 7c	Mice adults (welfare assessment)		6	
		Group 7d	Immune deficient mice adults (breeding)	200		2260

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Kon. Ned. Academie van Wetenschappen
[redacted]
Postbus 19121
1000 GC AMSTERDAM

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Postbus 20401
2500 EK Den Haag
www.zbo-ccd.nl
0900 28 000 28 (10 ct/min)

Onze referentie
Aanvraagnummer
AVD801002015125

Bijlagen
2

Datum 23-06-2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Geachte heer/mevrouw [redacted]
Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 22 juni 2015.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD801002015125. Gebruik dit nummer wanneer u contact met de CCD opneemt.

Wacht met de uitvoering van uw project
Als wij nog informatie van u nodig hebben dan ontvangt u daarover bericht. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn bijgeschreven op de rekening van de CCD. Zodra uw aanvraag compleet is, ontvangt u binnen veertig werkdagen een beslissing op uw aanvraag. In geval van een complexe aanvraag kan deze termijn met maximaal vijftien werkdagen verlengd worden. U krijgt bericht als de beslisperiode van uw aanvraag vanwege complexiteit wordt verlengd. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Factuur
Bijgaand treft u de factuur aan voor de betaling van de leges. Wij verzoeken u de leges zo spoedig mogelijk te voldoen, zodat we uw aanvraag in behandeling kunnen nemen. Is uw betaling niet binnen dertig dagen ontvangen, dan wordt uw aanvraag buiten behandeling gesteld. Dit betekent dat uw aanvraag niet beoordeeld wordt en u uw project niet mag starten.

Meer informatie
Heeft u vragen, kijk dan op www.zbo-ccd.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	80100
Naam instelling of organisatie:	Kon. Ned. Academie van Wetenschappen
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	54667089
Postbus:	19121
Postcode en plaats:	1000 GC AMSTERDAM
Tenaamstelling van het rekeningnummer:	Hubrecht Instituut/Nederlandshersen Instituur

Gegevens verantwoordelijke onderzoeker	
Naam:	[redacted]
Functie:	Group Leader
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Over uw aanvraag

Wat voor aanvraag doet u?	[x] Nieuwe aanvraag [ ] Wijziging op een (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn [ ] Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn

Over uw project

Geplande startdatum:	1 juni 2015
Geplande einddatum:	1 juni 2020
Titel project:	The molecular and cellular mechanisms of tumor initiation, growth, metastasis and thera
Titel niet-technische samenvatting:	Het begrijpen van het ontstaan en ontwikkeling van kanker, uitzaiingen en resistentie
Naam DEC:	DEC-KNAW
Postadres DEC:	[redacted] Amsterdam
E-mailadres DEC:	[redacted]

Betaalgegevens

De leges bedragen:	€ 741,-
De leges voldoet u:	na ontvangst van de factuur

Checklist bijlagen

Verplichte bijlagen:	[x] Projectvoorstel [x] Beschrijving Dierproeven [x] Niet-technische samenvatting
Overige bijlagen:	[x] DEC-advies

Ondertekening

Naam:	[redacted]
Functie:	Directeur Instituten KNAW
Plaats:	Amsterdam
Datum:	1 juni 2015

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD801002015125

Bijlagen
2

Datum 23-06-2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 23 juni 2015
Vervaldatum: 23 juli 2015
Factuurnummer: 201570125

Omschrijving	**Bedrag**
Betaling leges projectvegrunning dierproeven Betreft aanvraag AVD801002015125	€ 741,00

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL28RBOS 056.99.96.066 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 20401, 2500 EK te 's Gravenhage.

Format DEC-advies

Maak bij de toepassing van dit format gebruik van de bijbehorende toelichting, waarin elke stap in het beoordelingsproces wordt toegelicht

A. Algemene gegevens over de procedure

1. Aanvraagnummer: AVD/801002015125
2. Titel van het project: The molecular and cellular mechanisms of tumor initiation, growth, metastasis and therapy resistance.
3. Titel van de NTS: Het begrijpen van het ontstaan en ontwikkeling van kanker, uitzaaiingen en resistentie tegen therapie.
4. Type aanvraag:
   - ✓ nieuwe aanvraag projectvergunning
     wijziging van vergunning met nummer
5. Contactgegevens DEC:
   - naam DEC: KNAW
   - telefoonnummer contactpersoon: [redacted]
   - mailadres contactpersoon: [redacted]
6. Adviestraject (data dd-mm-jjjj):
   - ✓ ontvangen door DEC: 13-05-2015
   - ✓ aanvraag compleet: 15-06-2015
   - ✓ in vergadering besproken: 21-05-2015
   - ✓ anderszins behandeld: n.v.t.
   - ✓ termijnonderbreking(en): n.v.t.
     besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen:
     aanpassing aanvraag:
   - ✓ advies aan CCD: 22-06-2015
7. Eventueel horen van aanvrager
   - Datum: n.v.t.
   - Plaats:
   - Aantal aanwezige DEC-leden:
   - Aanwezige (namens) aanvrager:
8. Correspondentie met de aanvrager:
   - Datum 27-05-2015
   - Strekking: completering van de aanvraag
   - Datum antwoord: 15-06-2015
   - Strekking van de antwoorden: de aanvraag is gecompleteerd
9. Eventuele adviezen door experts (niet lid van de DEC): geen

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig. Het omvat dierproeven in de zin der wet.

2. De aanvraag betreft een nieuwe aanvraag. Er is enige overlap met een aantal al van een positief advies voorziene DEC-protocollen.
3. De DEC is competent om over deze projectvergunningsaanvraag te adviseren. De benodigde expertise op dit wetenschappelijk terrein is aanwezig binnen de DEC. Geen van de DEC-leden is betrokken bij het betreffende project.
4. Vanwege betrokkenheid bij het betreffende project is een aantal DEC-leden, met het oog op onafhankelijkheid en onpartijdigheid, niet betrokken bij de advisering: n.v.t.

C. Beoordeling (inhoud):

1. Het project is wetenschappelijk verantwoord.

2. De in de aanvraag aangekruiste doelcategorie is in overeenstemming met de hoofddoelstelling.

3. De doelstelling, in relatie tot de uitvoering, is helder omschreven; te weten het verkrijgen van fundamenteel wetenschappelijke inzichten in 1) de biologische mechanismen van processen die belangrijk zijn voor het ontstaan, groei, uitzaaiing en therapieresistentie van tumoren, en 2) het ontwikkelen/verbeteren van kankertherapieën. Op termijn kunnen de resultaten leiden tot nieuwe behandelingsmethoden voor patiënten met kanker.

   Het project richt zich op tumorvorming, het ontstaan van uitzaaiingen en therapieresistentie. Binnen dit project zijn de belangrijkste doelen het begrijpen wat de rol is van stamcellen en kankerstamcellen en wat de betrokkenheid is van de micro- en macro-omgeving waarin deze cellen zich bevinden. Het fundamenteel wetenschappelijke belang acht de DEC substantieel.

   Het verkrijgen van deze fundamenteel wetenschappelijke kennis is essentieel voor het ontwikkelen van nieuwe en/of verbeterde therapeutische strategieën voor de behandeling van kanker en dit is naar de mening van de DEC een substantieel belang. Het project dient hiermee een belangrijk maatschappelijk belang, gezien de grote groep patiënten met tumoren.

4. De gekozen strategie en experimentele aanpak in combinatie met de infrastructuur op het Hubrecht Instituut en de expertise van de betrokken onderzoeksgroep bieden een realistisch uitzicht op het behalen van de beoogde doelstellingen binnen gevraagde looptijd van het project. Het project bouwt voort op een langlopende lijn van onderzoek van een grote groep onderzoekers. Over de afgelopen jaren zijn met een vergelijkbare strategie en aanpak belangrijke wetenschappelijk resultaten behaald, resulterend in vele publicaties in vooraanstaande tijdschriften. Het onderzoek wordt financieel gesteund door verschillende onafhankelijke subsidiegevers. Er zijn internationale samenwerkingsverbanden met andere laboratoria actief in dit onderzoeksveld.

Verder zijn er nauwe banden met de kliniek waardoor er een sterke wisselwerking ontstaat tussen klinisch relevante vragen en het beschreven fundamentele onderzoek waar de dierproeven een deel van uit maken.

5. Alle dieren worden gefokt voor het gebruik in dierproeven, er is geen sprake van afwijkende huisvesting en/of hergebruik. Er is geen sprake van bedreigde diersoorten, niet-menselijke primaten, zwerfdieren en/of dieren in/uit het wild. De toegepaste methoden voor anesthesie/euthanasie zijn conform de Richtlijn.

6. Het cumulatieve ongerief gepaard gaand met de dierproeven, zoals beschreven in de zeven verschillende type dierproeven, is naar inschatting van de DEC licht (Type dierproef 1, 3, en 5) of matig (Type dierproef 2, 4, 6 en 7). Er een beperkt risico op onbedoelde bijwerkingen. Deze inschatting van de DEC is in overeenstemming met het niveau van cumulatief ongerief zoals dat is geclassificeerd door de onderzoekers. Dit is gebaseerd op hun ruime ervaring met de gebruikte modellen in vergelijkbare dierproeven.

7. Binnen het project wordt maximaal gebruik gemaakt van methoden die de voorgestelde dierproeven geheel of gedeeltelijk **vervangen.** Een belangrijk onderdeel van de experimentele strategie is de gefaseerde opzet. In de eerste fase, voorafgaand aan de dierproeven, vindt een uitgebreid onderzoek plaats met weefsel afkomstig van patiënten en cellijnen. Na deze fase zijn er go/no-go-beslissingsmomenten, voordat tot het uitvoeren van dierproeven wordt besloten. Nieuwe inzichten in de processen die de initiatie, groei en metastase van tumorcellen reguleren kunnen op dit moment alleen maar verkregen worden in een intact organisme. Deze processen, waarbij verschillende typen cellen betrokken zijn binnen een gecompliceerde anatomische context, zijn zeer complex en kunnen niet met cellijnen worden bestudeerd. Naar het oordeel van de DEC zijn er geen alternatieven beschikbaar voor het voorgestelde gebruik van intacte dieren om te doelstelling van dit project te realiseren.

8. In het project wordt optimaal tegemoet gekomen aan de vereisten van **vermindering** van dierproeven. De onderzoeksgroep heeft een jarenlange ervaring opgebouwd met dit soort experimenten en door een veelal gefaseerde opzet wordt per experiment niet meer dan het minimaal benodigde aantal dieren ingezet. Voorafgaand aan de kwantitatieve experimenten wordt op basis van literatuurgegevens, eigen historische data of een specifiek hiertoe uitgevoerd pilot experiment de groepsgrootte bepaald. Technieken en procedures worden zorgvuldig toegepast. Het aantal te gebruiken dieren is realistisch geschat.

9. De uitvoering van het project is in overeenstemming met de vereisten van **verfijning** van dierproeven en is zo opgezet dat de dierproeven met zo min mogelijk ongerief worden uitgevoerd.
Bij de opzet wordt rekening gehouden met dierenwelzijn en wel op de volgende manieren: 1) het gebruik van adequate anesthesie en analgesie waar nodig, 2)

een intensieve monitoring van de proefdieren na de inductie van tumoren, 3) het gebruik van weefselspecifiek genetisch-gemodificeerde muizen, 4) een monitoring op het optreden van onverwacht constitutioneel ongerief van nieuwe gecreëerde genotypes.
Er is geen sprake van belangwekkende milieueffecten.

10. De niet-technische samenvatting is een evenwichtige weergave van het project en is geformuleerd in begrijpelijke taal. De NTS voldoet daarmee aan de eisen zoals gesteld in artikel 10.a.1.7 van de Wod.

D. Ethische afweging

De centrale vraag voor de ethische afweging is of het belang van het doel van dit project opweegt tegen het ongerief dat de dieren ondergaan (geclassificeerd als licht of matig). Het doel van het project is het verkrijgen van fundamenteel wetenschappelijke inzichten in: 1) de biologische mechanismen van processen die belangrijk zijn voor het ontstaan, groei, uitzaaiing en therapieresistentie van tumoren, en 2) het ontwikkelen/verbeteren van kankertherapieën.
Het onderzoek is primair fundamenteel wetenschappelijk van karakter. De verwachting is dat de resultaten op den duur kunnen bijdragen aan nieuwe of verbeterde therapieën voor kankerpatiënten wat voor een grote groep patiënten van groot belang is om te overleven.

Het fundamenteel wetenschappelijke onderzoek in dit project is van aangetoonde en excellente kwaliteit. De onderzoeksgroep beschikt over ruime ervaring met de gekozen onderzoeksstrategie en met de zeven beschreven type dierproeven.

De classificatie van het ongerief van de dieren in de verschillende typen dierproeven is licht of matig. Bij de het uitvoeren van de dierproeven wordt een adequate invulling gegeven aan de vereisten op het gebied van de vervanging, vermindering en verfijning van de dierproeven. De DEC onderschrijft dat de doelstellingen niet zonder het gebruik van proefdieren kunnen worden behaald.

De DEC is van mening dat de resultaten van dierproeven zullen bijdragen aan het behalen van het geformuleerde doel en schat de kans op het realiseren van de fundamenteel wetenschappelijke doelstellingen in als hoog. Het project is uit wetenschappelijk oogpunt verantwoord. De verkregen fundamenteel wetenschappelijke kennis is essentieel om te kunnen komen tot nieuwe therapeutische benaderingen of van een verbetering van bestaande therapieën in patiënten met tumoren. Het gaat om een grote groep patiënten met uiteenlopende, op dit moment nog slecht behandelbare, aandoeningen. Het maatschappelijk belang is daarom groot.
De DEC komt tot de conclusie dat de doeleinden van het project het voorgestelde gebruik van de proefdieren en het daarmee samenhangende ongerief van de proefdieren rechtvaardigt.

E. Advies

1. Advies aan de CCD
   - ✓ **De DEC adviseert de vergunning te verlenen**
2. Het uitgebrachte advies is gebaseerd op consensus.
3. Er zijn geen knelpunten of dilemma's gesignaleerd tijdens het beoordelen van de aanvraag of het formuleren van het advies.

[redacted]

Van: secretariaat DEC [redacted]
Verzonden: maandag 22 juni 2015 10:08
Aan: ZBO-CCD
Onderwerp: AVD-801002015125 ingediend

Categorieën: [redacted]

Geachte CCD,

Als secretaris van de DEC-KNAW heb ik zojuist alle documenten behorende bij AVD-801002015125 naar u gestuurd via de webftp. Het getekende aanvraagformulier is afgelopen vrijdag per post naar u gestuurd.
Mochten er nog vragen zijn dan hoor ik dat graag.

Groet [redacted]
DEC-KNAW

[redacted]

Van: Info-zbo
Verzonden: woensdag 22 juli 2015 17:47
Aan: [redacted]
CC: [redacted]
Onderwerp: AVD801002015125: Aanvullende informatie

Geachte [redacted],

Op 22 juni 2015 heeft de CCD uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project “The molecular and cellular mechanisms of tumor initiation, growth, metasasis and therapy resistance”. De CCD heeft nog aanvullende informatie van u nodig om uw aanvraag verder te kunnen beoordelen.

-De DEC heeft in haar advies aangegeven dat er enige overlag is tussen de huidige vergunningaanvraag en eerder van een positief advies voorziene DEC protocollen. Om een beeld te krijgen van de mate van overlap en het aantal proefdieren dat daarmee gemoeid is, is het wenselijk antwoord te krijgen op de volgende vragen: In hoeverre is het noodzakelijk dat, daar waar er overlap is, de al eerder van een positief advies voorziene dierproeven en het huidige bij de CCD aangevraagde project naast elkaar uitgevoerd kunnen worden. Indien noodzakelijk, om welke in deze aanvraag beschreven dierproeven gaat het en hoeveel dieren zullen nog gebruikt worden op de DEC protocollen?

-De CCD hecht er aan dat het aantal dieren in voorraad gedood terug te dringen. In uw aanvraag beschrijft u niet of beide geslachten gebruikt kunnen worden. Indien u van plan bent alleen/voornamelijk muizen van 1 geslacht gaat gebruiken, kunt u onderbouwen waarom het belangrijk is dieren van 1 geslacht te gebruiken?

Opsturen informatie
U heeft 14 dagen de tijd om de ontbrekende informatie op te sturen. De CCD zou uw aanvraag echter graag tijdens haar eerstvolgende vergadering behandelen. De CCD zou de gevraagde informatie daarom uiterlijk maandag 27 juli 2015 van u ontvangen. U kunt deze informatie aanleveren via NetFTP of per e-mail.

Wanneer een beslissing
De beslistermijn op uw aanvraag wordt opgeschort tot het moment dat bovengenoemde informatie is ontvangen. Na ontvangst van uw reactie nemen wij uw aanvraag verder in behandeling. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Meer informatie
Heeft u vragen, kijk dan op www.zbo-ccd.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Bij voorbaat hartelijk dank,

Met vriendelijke groet,

[redacted]

Centrale Commissie Dierproeven **www.centralecommissiedierproeven.nl**

Postbus 20401 | 2500 EK | Den Haag

T: 0900 2800028
E: info@zbo-ccd.nl (Let op: nieuw e-mail adres)

Hubrecht Institute

Developmental Biology
and Stem Cell Research

Centrale Commissie Dierproeven
Postbus 20401
2500EK Den Haag

Datum: Utrecht, 24 juli 2015.
Uw referentie: AVD80100-2015-125

Geachte Leden van de CCD,

Deze brief schrijven wij u n.a.v. uw brief/email d.d. 22 juli 2015. In deze brief verzocht u ons om aanvullende informatie nodig om projectvergunning dierproeven (AVD80100-2015-125) verder te kunnen beoordelen. Hierbij voldoen wij aan dit verzoek.

- De DEC heeft in haar advies aangegeven dat er enige overlag is tussen de huidige vergunningaanvraag en eerder van een positief advies voorziene DEC protocollen. Om een beeld te krijgen van de mate van overlap en het aantal proefdieren dat daarmee gemoeid is, is het wenselijk antwoord te krijgen op de volgende vragen: In hoeverre is het noodzakelijk dat, daar waar er overlap is, de al eerder van een positief advies voorziene dierproeven en het huidige bij de CCD aangevraagde project naast elkaar uitgevoerd kunnen worden. Indien noodzakelijk, om welke in deze aanvraag beschreven dierproeven gaat het en hoeveel dieren zullen nog gebruikt worden op de DEC protocollen?

Antwoord vraag 1:
Om de continuïteit van ons onderzoek te garanderen en omdat een aantal op dit moment lopende goedgekeurde DEC protocollen allemaal in een verschillend stadium van uitvoering zijn is het onvermijdelijk dat er een overlap is tussen deze projectaanvraag en de lopende DEC protocollen. Na het verlenen van de projectvergunning door de CCD zullen al onze proefdieren en dierexperimenten formeel gaan vallen onder deze vergunning en zullen de dieren en de experimenten 'afgeschreven' worden van de beschrijvingen en aantallen in projectbeschrijving.

-De CCD hecht er aan dat het aantal dieren in voorraad gedood terug te dringen. In uw aanvraag beschrijft u niet of beide geslachten gebruikt kunnen worden. Indien u van plan bent alleen/voornamelijk muizen van 1 geslacht gaat gebruiken, kunt u onderbouwen waarom het belangrijk is dieren van 1 geslacht te gebruiken?

Antwoord vraag 2:
In onze beschreven experimenten maken we, waar mogelijk, geen onderscheid tussen beide geslachten en zullen zowel mannetjes als vrouwtjes gebruikt worden. Dit zal resulteren in een vermindering van het

Hubrecht Institute

Developmental Biology
and Stem Cell Research

aantal in voorraad gedode dieren. Uitzondering hierop zijn studies van tumoren die geslachts-afhankelijk zijn zoals borstkanker.

Wij hopen U hiermee voldoende geïnformeerd te hebben en zien uw reactie met belangstelling tegemoet.

Hoogachtend,

[redacted]

[redacted] - KNAW

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

KNAW
[redacted]
Postbus 19121
1000GC Amsterdam

Centrale Commissie Dierproeven

Postbus 20401
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www.centralecommissiedierproeven.nl

T 0900-28 000 28 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD801002015125

Uw referentie

Bijlagen
1

Datum 10-08-2015
Betreft Beslissing Aanvraag projectvergunning dierproeven

Geachte [redacted]

Op 22 juni 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "The molecular and cellular mechanisms of tumor initiation, growth, metasasis and therapy resistance" met aanvraagnummer AVD801002015125. Wij hebben uw aanvraag beoordeeld.

Op 24 juli 2015 heeft u uw aanvraag aangevuld na vragen van het secretariaat.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de dierproeven (hierna de wet), voor de periode van 12 augustus 2015 tot 01 juni 2020. Hierbij gelden de voorwaarden zoals genoemd in de vergunning. De looptijd van de vergunning wijkt af van de aangevraagde periode omdat de aangevraagde startdatum van het project in het verleden ligt. U kunt met uw project "The molecular and cellular mechanisms of tumor initiation, growth, metasasis and therapy resistance" starten.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC KNAW gevoegd d.d. 22 juni 2015. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a lid 3 van de wet. Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Wij nemen dit advies van de commissie over, inclusief de daaraan ten grondslag liggende motivering. Naar aanleiding van de door de DEC gesignaleerde overlap tussen de huidige vergunningsaanvraag en eerder van een positief advies voorziene DEC protocollen, is de voorwaarde toegevoegd dat daar waar er sprake is van overlap tussen de in deze vergunning vergunde dierproeven en eerder goedgekeurde DEC protocollen de dieren en experimenten na het verlenen van de vergunning formeel onder deze vergunning gaan vallen, zoals u in uw brief van 24 juli 2015 heeft aangegeven. Hierdoor is er geen sprake meer van overlap.

Het DEC advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving liggen ten grondslag aan dit besluit.

Bezwaar
Als u het niet eens bent met deze beslissing, kunt u binnen zes weken na verzending van deze brief schriftelijk een bezwaarschrift indienen.
Een bezwaarschrift kunt u sturen naar Centrale Commissie Dierproeven, afdeling Juridische Zaken, postbus 20401, 2500 EK Den Haag.

Bij het indienen van een bezwaarschrift vragen we u in ieder geval de datum van de beslissing waartegen u bezwaar maakt en het aanvraagnummer te vermelden. U vindt deze gegevens in het colofon.

Bezwaar schorst niet de werking van het besluit waar u het niet mee eens bent. Dat betekent dat dat besluit wel in werking treedt en geldig is. U kunt tijdens deze procedure een voorlopige voorziening vragen bij de Voorzieningenrechter van de rechtbank in de woonplaats van de aanvrager. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisend belang.

Voor de behandeling van een voorlopige voorziening is griffierecht verschuldigd. Op http://www.rechtspraak.nl/Organisatie/Rechtbanken/Pages/default.aspx kunt u zien onder welke rechtbank de vestigingsplaats van de aanvrager valt.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

De Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Peuter
Algemeen Secretaris

Dit besluit is genomen met inachtneming van het Besluit mandaat, volmacht en machtiging van de Centrale Commissie Dierproeven CCD 2014 zoals de Centrale Commissie Dierproeven heeft vastgesteld op 19 december 2014, ref 2014-04 en is gepubliceerd in de Staatscourant van 2 januari 2015, Nr. 163

Bijlagen
- Vergunning
  Hiervan deel uitmakend:
  - DEC-advies
  - Weergave wet- en regelgeving

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de dierproeven

Verleent de Centrale Commissie Dierproeven aan
Naam: KNAW
Adres: Postbus 19121
Postcode en woonplaats: 1000GC Amsterdam
Deelnemersnummer: 80100

deze projectvergunning voor het tijdvak 12 augustus 2015 tot 01 juni 2020, voor het project "The molecular and cellular mechanisms of tumor initiation, growth, metasasis and therapy resistance" met aanvraagnummer AVD801002015125, gebaseerd op het advies van Dierexperimentencommissie DEC KNAW.
De functie van de verantwoordelijk onderzoeker is Group Leader.
De aanvraag omvat de volgende bescheiden:

1. een aanvraagformulier projectvergunning dierproeven, ontvangen op 22 juni 2015
2. de bij het aanvraagformulier behorende bijlagen:
    a. Projectvoorstel, zoals ontvangen bij digitale indiening op 22 juni 2015 en brief ontvangen op 24 juli 2015;
    b. Niet-technische Samenvatting van het project, zoals ontvangen bij digitale indiening op 22 juni 2015;
    c. Advies van Dierexperimentencommissie d.d. 22 juni 2015, ontvangen op 22 juni 2015;
    d. Aanvullende informatie ontvangen op 24 juli 2015.

Dierproeven

Naam dierproef	**Diersoort**	**Aantal dieren**	**Ernst**
Gene (in) activation interventions (ex vivo analysis)	Muis (WT, GGM en mutanten) Embryo's Neonaten Volwassenen	 200 100 4000	 Licht Licht Licht: 96% Matig <1 dag: 1% Matig continu: <3%
Gene (in) activation interventions (in vivo imaging)	Muis (WT, GGM en mutanten) Volwassenen	 5000	 Licht: 15% Matig: 85%
Transplantation (ex vivo analysis)	Muis (WT, GGM en mutanten) Volwassenen	 4500	 Licht: 96% Matig <1 dag: 1% Matig continu: <3%
Transplantation (in vivo imaging)	Muis (WT, GGM en mutanten) Volwassenen	 2000	 Licht: 12% Matig: 88%
Transplantation Gene (in) activation interventions (ex vivo analysis)	Muis (WT, GGM en mutanten) Volwassenen	 1650	 Licht: 96% Matig <1 dag: 1% Matig continu: <3%
Transplantation Gene (in) activation interventions (in vivo imaging)	Muis (WT, GGM en mutanten)		

	Volwassenen	2800	Matig
Generation Welfare assessment and Breeding of GMM	Muis (WT en GGM) Volwassenen: Donoren Foster dieren GGO dieren Immuun deficiënte dieren Verklikkerdieren	2260	 Matig Matig Geen: 99% Licht: 1% Geen Geen

Voorwaarden
Op grond van artikel 10a1 lid 2 Wet zijn aan een projectvergunning voorwaarden te stellen

De vergunning wordt verleend onder de voorwaarde dat daar waar er sprake is van overlap tussen de in deze vergunning vergunde dierproeven en eerder goedgekeurde DEC protocollen zullen de dieren en experimenten na het verlenen van de vergunning formeel onder deze vergunning gaan vallen, zoals de aanvrager in zijn brief van 24 juli 2015 ook heeft aangegeven. Hierdoor is er geen sprake meer van overlap.

In artikel 10, lid 1a van de wet, wordt bepaald dat het verboden is een dierproef te verrichten voor een doel dat, naar de algemeen kenbare, onder deskundigen heersende opvatting, ook kan worden bereikt anders dan door middel van een dierproef, of door middel van een dierproef waarbij minder dieren kunnen worden gebruikt of minder ongerief wordt berokkend dan bij de in het geding zijnde proef het geval is. Nieuwe onderzoeken naar alternatieven kunnen tot gevolg hebben dat inzichten en/of omstandigheden van het aangevraagde project in de vergunningsperiode wijzigen, gedurende de looptijd van deze vergunning. Indien bovenstaande zich voordoet dient aanvrager dit in overleg met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarden wijzigen of intrekken.

Weergave wet- en regelgeving

Dit project en wijzigingen

Volgens artikel 10c van de Wet op de dierproeven (hierna de wet) is het verboden om andere dierproeven uit te voeren dan waar de vergunning voor is verleend. De dierproeven mogen slechts worden verricht in het kader van een project, volgens artikel 10g. Uit artikel 10b volgt dat de dierproeven zijn ingedeeld in de categorieën terminaal, licht, matig of ernstig. Als er wijzigingen in een dierproef plaatsvinden, moeten deze gemeld worden aan de Centrale Commissie Dierproeven. Hebben de wijzigingen negatieve gevolgen voor het dierenwelzijn, dan moet volgens artikel 10a5 de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven.
Artikel 10b schrijft voor dat het verboden is een dierproef te verrichten die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, tenzij hiervoor door de Minister van Economische Zaken een ontheffing is verleend.

Verzorging

De fokker, leverancier en gebruiker moeten volgens artikel 13f van de wet over voldoende personeel beschikken en ervoor zorgen dat de dieren behoorlijk worden verzorgd, behandeld en gehuisvest. Er moeten ook personen zijn die toezicht houden op het welzijn en de verzorging van de dieren in de inrichting, personeel dat met de dieren omgaat moet toegang hebben tot informatie over de in de inrichting gehuisveste soorten en personeel moet voldoende geschoold en bekwaam zijn. Ook moeten er personen zijn die een eind kunnen maken aan onnodige pijn, lijden, angst of blijvende schade die tijdens een dierproef bij een dier wordt veroorzaakt. Daarnaast zijn er personen die zorgen dat een project volgens deze vergunning wordt uitgevoerd en als dat niet mogelijk is zorgen dat er passende maatregelen worden getroffen.
In artikel 9 staat dat de persoon die het project en de dierproef opzet deskundig en bekwaam moet zijn.
In artikel 8 van het Dierproevenbesluit 2014 staat dat personen die dierproeven verrichten, de dieren verzorgen of de dieren doden, hiervoor een opleiding moeten hebben afgerond.
Voordat een dierproef die onderdeel uitmaakt van dit project start, moet volgens artikel 10a3 van de wet de uitvoering afgestemd worden met de instantie voor dierenwelzijn.

Pijnbestrijding en verdoving

In artikel 13 van de wet staat dat een dierproef onder algehele of plaatselijke verdoving wordt uitgevoerd tenzij dat niet mogelijk is, dan wel bij het verrichten van een dierproef worden pijnstillers toegediend of andere goede methoden gebruikt die de pijn, het lijden, de angst of de blijvende schade bij het dier tot een minimum beperken. Een dierproef die bij het dier gepaard gaat met zwaar letsel dat hevige pijn kan veroorzaken, wordt niet zonder verdoving uitgevoerd. Hierbij wordt afgewogen of het toedienen van verdoving voor het dier traumatischer is dan de dierproef zelf en het toedienen van verdoving onverenigbaar is met het doel van de dierproef. Bij een dier wordt geen stof toegediend waardoor het dier niet meer of slechts in verminderde mate in staat is pijn te tonen, wanneer het dier niet tegelijkertijd voldoende verdoving of pijnstilling krijgt toegediend, tenzij wetenschappelijk gemotiveerd. Dieren die pijn kunnen lijden als de verdoving eenmaal is uitgewerkt, moeten preventief en postoperatief behandeld worden met pijnstillers of andere geschikte pijnbestrijdingsmethoden, mits die verenigbaar zijn met het doel van de dierproef. Zodra het doel van de dierproef is bereikt, moeten passende maatregelen worden genomen om het lijden van het dier tot een minimum te beperken.

Einde van een dierproef

Artikel 13a van de wet bepaalt dat een dierproef is afgelopen wanneer voor die dierproef geen verdere waarnemingen hoeven te worden verricht of, voor wat betreft nieuwe genetisch gemodificeerde dierenlijnen, wanneer bij de nakomelingen niet evenveel of meer, pijn, lijden, angst, of blijvende schade wordt waargenomen of verwacht dan bij het inbrengen van een naald. Er wordt dan door een dierenarts of een andere ter zake deskundige beslist of het dier in leven zal worden gehouden. Een dier wordt gedood als aannemelijk is dat het een matige of ernstige vorm van pijn, lijden, angst of blijven schade zal blijven ondervinden. Als een dier in leven wordt gehouden, krijgt het de verzorging en huisvesting die past bij zijn gezondheidstoestand.
Volgens artikel 13b moet de dood als eindpunt van een dierproef zoveel mogelijk worden vermeden en vervangen door in een vroege fase vaststelbare, humane eindpunten. Als de dood als eindpunt onvermijdelijk is, moeten er zo weinig mogelijk dieren sterven en het lijden zo veel mogelijk beperkt blijven.

Uit artikel 13c volgt dat het doden van dieren door een deskundig persoon moet worden gedaan, wat zo min mogelijk pijn, lijden en angst met zich meebrengt. De methode om te doden is vastgesteld in de Europese richtlijn artikel 6.
In artikel 13d is vastgesteld dat proefdieren geadopteerd kunnen worden, teruggeplaatst in hun habitat of in een geschikt dierhouderijsysteem, als de gezondheidstoestand van het dier het toelaat, er geen gevaar is voor volksgezondheid, diergezondheid of milieu en er passende maatregelen zijn genomen om het welzijn van het dier te waarborgen.