Tools and techniques for Wt1-based lineage tracing

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i. Summary / Abstract

The spatiotemporal expression pattern of Wt1 has been extensively studied in a number of animal models to establish its function and the developmental fate of the cells expressing this gene. In this chapter, we review the available animal models for Wt1-expressing cell lineage analysis, including direct Wt1 expression reporters and systems for permanent Wt1 lineage tracing. We describe the presently used constitutive or inducible genetic lineage tracing approaches based on the Cre/loxP system utilising Cre recombinase expression under control of a Wt1 promoter.

To make these systems accessible, we provide laboratory protocols that include dissection and processing of the tissues for immunofluoresence and histopathological analysis of the lineage-labelled Wt1-derived cells within the embryo/tissue context.

ii. Key words

Wt1, cell lineage tracing, Cre/loxP, inducible Cre recombinase, Cre-ERT2, Tamoxifen, Rosa26 reporter mice

1. Introduction

A number of murine models are available to study both, Wt1-expressing cell lineage and actual Wt1 expression. The first models are based on a Cre/loxP system, inducing constitutive expression of a reporter gene in those cells where the Wt1 promoter controls expression of the Cre-recombinase enzyme. Different levels of recombination can produce different levels of reporter expression, and rigorous

controls using Cre-/-; Flox+/+ embryos must be performed to discard events of spontaneous recombination. On the other hand, direct activation of a reporter gene by the Wt1 promoter allows to directly monitor Wt1 expression in the tissues. The replacement of the exon 1 of the Wt1 gene by a GFP expressing system (Wt1 knock-in system, 1) also allows for detection of Wt1 expression in heterozygous ($Wt1^{+/GFP}$) embryos using the original Wt1 promoter (see below).

Direct Wt1 expression reporters:

Wt1-expressing cells in tissues can be analysed using in situ hybridisation and immunohistochemical approaches. However, a set of mouse lines that function as direct Wt1-LacZ or-GFP reporters, enable detection of cells that are expressing Wt1 at any given time (2-5). The Wt1-LacZ reporters were generated initially by yeast recombineering of YAC clones containing the human WT1 locus with a LacZ-containing cassette, before pronuclear injections of the resulting YAC constructs gave rise to a range of transgenic LacZ reporters (Tg(WT1)HNdh, Tg(WT1)WANdh, Tg(WT1)WCNdh, Tg(WT1)WWNdh; Refs. 2, 3). Analysis of these transgenic Wt1-LacZ reporter lines revealed a highly faithful recapitulation of endogenous Wt1 expression in a range of embryonic tissues, and some degree of rescue function by combination with $Wt1^{-/-}$ mutants.

By contrast, the $Wt1^{tm1Nhsn}$ reporter mouse line was generated as knock-in of a GFP-cassette containing construct by gene targeting, thus replacing exon1 and part of the intronic region in the resulting mouse line $Wt1^{tm1Nhsn}$ (5). In $Wt1^{tm1Nhsn}$ heterozygous mice, GFP is expressed directly under control of the endogenous Wt1 promoter elements, while $Wt1^{tm1Nhsn}$ homozygous mice are embryonic lethal due to the

inactivation of the Wt1 locus at the GFP insertion site. The GFP expression can be observed directly under the dissecting microscope (Figure 1A) or by confocal microscopy, but the signal can also be much enhanced by GFP immunolocalization (Figure 1B).

The GFP reporter line $Wt1^{tm1(EGFP/cre)Wtp}$ was generated following a similar gene targeting strategy, and also results in GFP expression under direct control of the endogenous Wt1 promoter. However, in this mouse line, GFP is fused with the Cre recombinase protein, providing these mice with dual function for direct labelling of Wt1 expression, and as tool for lineage tracing studies (6). Both mouse lines have been proven useful as tools to study Wt1 expression in embryonic tissues, but also in isolated cells *in vitro*. Two further transgenic mouse lines which carry eGFP in combination with Cre recombinase under control of a Wt1 promoter have been described (Tg(Wt1-cre)#Jbeb, Ref. 7; Tg(Wt1-EGFP/cre)1Akis, Ref. 8), but their use as direct reporters for Wt1-controlled GFP expression have not been demonstrated. As shown below, GFP expression in Tg(Wt1-cre)#Jbeb animals is below the detection limit.

Permanent Wt1 lineage tracing – constitutive Cre recombinase expressed under control of Wt1:

Six different mouse lines have been described that utilise constitutive Cre recombinase expression under control of the mouse or the human WT1 gene for permanent lineage tracing (Figure 2):

- Wt1-Cre, first described by the Bader lab (9) (official nomenclature [1]: Tg(WT1-cre)AG11Dbdr; MGI:3609978)
- mWt1/IRES/GFP-Cre or *Wt1*^{Cre}, generated by the Burch lab (7) (official nomenclature: Tg(Wt1-cre)#Jbeb; MGI:5308608)
- Wt1^{GFPCre}, generated by the Pu lab (6) (official nomenclature: Wt1$^{tm1(EGFP/cre)Wtp}$; MGI:3801681)
- Wt1$^{BAC\text{-}IRES\text{-}EGFPCre}$, generated by the Kispert lab (8) (official nomenclature: Tg(Wt1-EGFP/cre)1Akis; MGI:5002800)
- WT1(RP23-8C14)-Cre, generated by the Burch lab (10) (official nomenclature: Tg(Wt1-cre)1Jbeb; MGI:5562908)

Similar protocols for permanent lineage tracing of Wt1-expressing cells have been described for all mouse lines of this category. Here, we detail the lineage tracing protocol using the *mWt1/IRES/GFP-Cre* (*Wt1*^{Cre}) mouse line.

The *Wt1*^{Cre} mouse line was developed using a BAC recombineering strategy to insert an IRES/EGFP-CRE cassette 17 bp downstream of the translation stop site of the Wt1 gene in the BAC clone RP23-266M16. The resultant recombinant BAC clone was used to generate several independent transgenic mouse lines that express *Cre* in the epicardial lineage beginning at the proepicardial stage. These *Wt1*^{Cre} transgenic mice have been used in previous studies to trace or delete specific genes in *Wt1*-expressing cells (7, 11, 12, 13). Crossing of homozygote (*Wt1*$^{Cre+/+}$) mice with B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J mice (Rosa26R-EYFP in short) allows to generate permanent reporter expression in *Wt1*-expressing cells (Figure 3A,B).

[1]Official nomenclature of mouse lines can be found under the Jackson laboratory website: www.informatics.jax.org

Of note, the Cre driver used for these studies induces the expression of low levels of GFP, but this expression does not interfere with the YFP expression of the reporter. Control $Wt1^{Cre+/+}$; Rosa26R-$EYFP^{-/-}$ embryos show no detectable GFP levels in the embryonic tissues by confocal microscopy (Figure 3C,D).

Temporal control of Wt1 lineage tracing - inducible Cre recombinase expressed under control of Wt1:

So far, one mouse line has been described that allows temporal induction of Cre recombinase activity under control of *Wt1* transcriptional regulation:

- $Wt1^{CreERT2}$, generated in the Pu lab (6) (official nomenclature: $Wt1^{tm2(cre/ERT2)Wtp}$; MGI:3801682).

The $Wt1^{CreERT2}$mouse line was generated by gene targeting a construct containing the Cre-ERT2 recombinant expression cassette in alignment with the start codon, thus replacing the first coding exon. In the $Wt1^{CreERT2}$ mouse line, Cre recombinase activity is induced by administration of Tamoxifen to mice. The Cre recombinase in these mice is fused to a modified estrogen receptor (ERT2; Ref. 14). The Cre-ERT2 fusion protein can only enter the nucleus when binding of Tamoxifen to the ERT2 part of the fusion protein has led to a conformational change; in the absence of Tamoxifen, the Cre-ERT2 fusion protein is localised in the cytoplasm. In mice carrying the $Wt1^{CreERT2}$ allele and a genomic region flanked by loxP recognition sites, the Cre recombinase can remove the loxP-flanked elements after Tamoxifen administration.

Because Tamoxifen is required for the activation of Cre recombinase activity, it allows for temporal control of reporter gene activity. Thus, the system enables the analysis of the contribution and fate of Wt1-expressing cells at specific, defined time

points, either in embryonic development, or in post-natal stages. Tamoxifen as the inducer of Cre recombination acts as a pulse, which results in irreversible activation of the reporter expression only when Tamoxifen has led to the Cre-ERT2 conformational change. The time that passes between Tamoxifen-induced recombination ('pulse') and time point of analysis represents the 'chase' (Figure 4): cells that change their fate and/or position within the tissue through differentiation and/or migration, may downregulate Wt1 and Cre expression, but will still be detectable for the reporter expression. Assessment of change in fate over time can be performed using immunohistochemistry with differentiation-specific markers.

$Wt1^{CreERT2}$ mice have been successfully used for lineage tracing studies in combination with Rosa26R-LacZ (15, 16), Rosa26R-EYFP (17) or Rosa26R-mTmG mice (4, 6, 17, 18, Wilm et al., in preparation; Figure 5). For more information on Cre-specific reporter mouse strains, we refer to the Jackson laboratory website (http://cre.jax.org/crereporters.html).

Most applications using the $Wt1^{CreERT2}$;reporter system involve embryonic lineage tracing studies where Tamoxifen is administered once at a specific time point during development (4, 6, 19-22). However, in studies where adult Wt1-expressing cells are traced, either in unchallenged or injured animals, Tamoxifen is usually administered on several occasions within 1-2 weeks, followed by a wash-out period (23, 24, Wilm et al., in preparation).

2. Materials

Solutions for histological analysis are prepared from analytical grade chemicals with autoclaved destilled water. Solutions are autoclaved, where appropriate, and stored at room temperature, 4 °C or -20 °C.

2.1 Tamoxifen solution and administration

1. 40 mg/ml Tamoxifen solution: weigh 1 g Tamoxifen (for example: T5648, Sigma) into a 50 ml universal tube and add 2.5 ml of 100% Ethanol (molecular grade). Mix thoroughly and then add 22.5 ml corn oil (for example: C8267, Sigma). Transfer solution to small bottle wrapped in foil, add a small stir bar and store at 4 °C. (see Note 1).

2. Oral gavage needle (available in a range of sizes from different companies)

2.2 Preparation of tissue for frozen sections

1. 10x PBS stock solution: weigh in 80 g $NaCl_2$, 2 g KCl, 14.4 g Na_2HPO_4 and 2.4 g KH_2HPO_4 in a 2 l beaker, and add 800 ml destilled H_2O. Dissolve using a stir bar on a magnetic stirrer, and adjust pH to 7.4 using concentrated HCl. Once pH is adjusted, transfer solution to 1000 ml graduated cylinder and add distilled H_2O to make 1000 ml. Fill into a clean blue-capped bottle, autoclave and store at room temperature.

2. Phosphate Buffered Saline (PBS): either dilute 10x PBS stock solution to 1x working solution, or make up from PBS tablets (for example: P4417, Sigma), using destilled, autoclaved H_2O.

3. 4% Paraformaldehyde (PFA) solution: weigh in 10 g PFA and dissolve in 200 ml destilled H_2O and 25 ml 10x PBS, under constant stirring and by slowly warming up the solution to maximum of 60°C, in the fume cupboard. Avoid breathing in the toxic fumes! Once solution has cleared, make solution up to 250 ml using destilled H_2O in a measuring cylinder. Transfer 4% PFA solution into a clean bottle, autoclave and store at 4 °C, or aliquot and store at -20 °C.

4. 15% sucrose in PBS: weigh in 15 g Sucrose and dissolve in 80 ml PBS; rotating and warming helps the sucrose to dissolve. Once dissolved, make solution up to 100 ml using PBS in a measuring cylinder, aliquot in 15 ml universal tube and store at -20 °C (see Note 3).

5. 30% sucrose in PBS: weigh in 30 g Sucrose and dissolve in 60 ml PBS; rotating and warming helps the sucrose to dissolve. Once dissolved, make solution up to 100 ml using PBS in a measuring cylinder, aliquot in 15 ml universal tube and store at -20 °C (see Note 3).

6. Optimal cutting temperature compound (OCT; Tissue Tek or other suppliers)

7. Cryostat including anti-roll blade, cryoblades

8. Superfrost Plus glass slides (Thermo Scientific or other suppliers)

9. Isopentane

2.3 Immunofluorescence analysis

1. Tris-PBS (TPBS): 1x TPBS solution: Weigh 7 g $NaCl_2$, 1.48 g Na_2HPO_4 and 043 g KH_2PO_4 and 5 g of Trizma base (Sigma, T1503) in a 2 l beaker, and add 800 ml

destilled H_2O. Dissolve using a stir bar on a magnetic stirrer, and adjust pH to 7.8 using concentrated HCl. Once pH is adjusted, transfer solution to 1000 ml graduated cylinder and add distilled H_2O to make 1000 ml.

2. Serum blocking solution (SB): 16% sheep serum, 1% bovine albumin in TPBS

3. Serum blocking solution with Triton (SBT): 16% sheep serum, 1% bovine albumin, 0.1% Triton X-100 in TPBS

4. Avidin-Biotin blocking kit (Vector)

5. Monovalent donkey anti-mouse IgG, Fab fragment (Jackson)

6. Primary antibodies: available from a plethora of companies

7. Secondary antibodies: take note to match the fluorophore with the filter sets available if using an epifluorescent or confocal microscope

8. 5 mg/ml DAPI stock solution: dissolve 5 mg DAPI (for example: D9542, Sigma) in 1 ml molecular grade H_2O. Complete dissolution can take some time. Store at -20 °C.

9. 1 μg/ml DAPI working solution: dilute 1 μl DAPI stock solution in 5 ml PBS. This solution can be directly added to the sections.

10. Mounting medium: a 1:1 glycerol:PBS solution can be used for temporal mounting. Alternatively, commercial mounting media are available (eg. Gel Mount from Biomeda or Sigma).

11. Humid chamber: A closed box containing a horizontal surface covered by filter paper moistened in distilled water.

2.4 X-gal staining on frozen sections

1. 40 mg/ml X-gal stock solution: Dilute 20 mg of X-gal (for example: Boehringer Mannheim, #745-740) in 0.5 ml of Dimethylformamide (for example: 227056, Sigma). Mix well and store at –20°C in a 1 ml tube protected from light.

2. 100 mM KFerro II solution: Weigh in 2.11 g Potassium hexacyanoferrate(II) trihydrate (for example: P3289, Sigma) into a 50 ml universal container and dissolve in 50 ml destilled, autoclaved H_2O. Wrap in foil to protect from light and store at room temperature.

3. 100 mM KFerro III solution: Weigh in 1.65 g Potassium hexacyanoferrate(III) (for example: 244023, Sigma) into a 50 ml universal container and dissolve in 50 ml destilled, autoclaved H_2O. Wrap in foil to protect from light and store at room temperature.

4. 1 M $MgCl_2$ solution: Weigh in 203.3 g $MgCl_2$ hexahydrate into a 1.5 l glass beaker and dissolve in 800 ml destilled H_2O under stirring. Using a 1000 ml measuring cylinder, fill solution up to 1000 ml with destilled H_2O, fill into blue-capped bottle and autoclave. Store at room temperature.

5. X-gal dilution buffer for frozen sections: In a 50 ml universal container, add 500 µl 100 mM KFerro II solution, 500 µl 100 mM KFerro III solution, 1 ml 10x PBS and 20 µl 1 M $MgCl_2$ to 7730 µl destilled H_2O and mix well.

6. X-gal Working Solution: Warm X-gal dilution buffer to 37°C and then add 250 µl 40 mg/ml X-gal stock solution. Use immediately and discard any leftover solution.

7. Neutral Red Solution. Weigh 3.3 g neutral red and dissolve in 1000 ml destilled

H_2O under stirring. Commercial solutions are available (For example: N2889, Sigma).

8. Eukitt mounting medium (available from different suppliers).

2.5 X-gal staining of tissues

1. 0.2% Glutaraldehyde/2% PFA fixative for whole mount X-gal staining: In a 50 ml universal container, mix 400 µl 25% Glutaraldehyde (grade II, for example G6257 from Sigma) and 25 ml 4% PFA solution with 24.6 ml PBS. Make fresh before each use (see Note 4).

2. 20 mg/ml X-gal stock solution: Dilute 20 mg of X-gal (for example: Boehringer Mannheim, #745-740) in 1 ml of Dimethylformamide (for example: 227056, Sigma). Mix well and store in a 1 ml tube protected from light at –20°C.

3. 10% Nonidet P-40 solution: Dilute 1 ml Nonidet P-40 (IGEPAL CA-630, I3021, Sigma) in 9 ml of destilled, autoclaved H_2O in a 15 ml universal container, warm up slightly to aid the detergent to dissolve, and store at room temperature (see Note 5).

4. 1% Sodium deoxycholate (NaDOC) solution: Weigh in 500 mg of NaDOC (for example: D6750, Sigma) into a 50 ml universal container and dissolve in 50 ml of destilled, autoclaved H_2O. Store at room temperature.

5. Wash solution: Add 100 µl 10% Nonidet P-40 to 50 ml PBS to give a final concentration of 0.02% Nonidet P-40 in PBS.

6. X-gal staining solution: Mix together in a 50 ml universal container 2.5 ml 100 mM KFerro II solution, 2.5 ml 100 mM KFerro III solution, 5 ml 10x PBS, 100 µl 1

M $MgCl_2$, 100 µl 10% Nonidet P-40 solution and 500 µl 1% NaDOC solution with 36.8 ml destilled H_2O and mix well. Add 2.5 ml 20mg/ml X-gal solution and use immediately; discard any leftover solution.

2.6 Eosin-counterstained paraffin sections of X-gal stained tissue

1. Isopropanol / Isopropyl alcohol (for example: W292907, Sigma)

2. Paraffin pellets (various suppliers)

3. Isopropanol:Paraffin solution: In a prewarmed 100 ml glass bottle, mix 50 ml Isopropanol with 50 ml liquid paraffin, stir and keep in a histology oven at around 58–60 °C until further use.

4. Histology oven, embedding station, microtome, microtome blades, histology water bath, stirrer

5. Embedding cassettes (various suppliers) or Peel-A-Ways embedding molds (for example: 18986 or 18646A, Polysciences)

5. Xylene, histology grade (for example: 534056, Sigma) or Histoclear (for example: HS-200, National Diagnostics)

6. Superfrost Plus glass slides (Thermo Scientific or other suppliers)

7. Glass slide racks with metal handle and staining dish (for example: 70312-20, EMS)

8. 1% Eosin Y stock solution: Weigh in 10 g Eosin Y (for example: E4009, Sigma), place in a 250 ml bottle, and dissolve in 200 ml destilled, autoclaved H_2O. Add 800

ml 95-100% Ethanol to generate a 1% Eosin Y stock solution. Store at room temperature.

9. 0.25% Eosin Y staining solution: Dilute the Eosin Y stock solution further by adding 750 ml of 80% Ethanol to 250 ml Eosin Y stock solution. Add 5 ml glacial acetic acid and mix well. Store at room temperature.

10. DPX mountant for histology (06522, Sigma)

11. Cover slip Best No.1, 22 mm x 50 mm (12342118, Thermo Scientific)

3. Methods

3.1 Tamoxifen administration

1. Gently warm the Tamoxifen solution on a heated stirrer until completely liquid.

2. Intraperitoneal injection, subcutaneous injection and oral gavage have been reported as successful administration routes (6,23,25). The optimal route of administration depends on a range of factors that need to be considered for each specific experimental design; factors to consider are deposition and leakage of oil after subcutaneous injection; interaction of oil with Wt1-expressing, mesothelial tissues after intraperitoneal injection; and feasibility of oral gavage (25; BW, unpublished observations).

3. Typically, the doses for Tamoxifen administration range between 1-2 mg Tamoxifen per 10 g body weight. Dosing regimens depend on the experimental design and range from once at a specific time point during embryonic development in case of embryonic lineage tracing, to 2-5 times within a week for postnatal or adult

lineage tracing (see Note 6). The pharmacological half-life of Tamoxifen after a single dose of Tamoxifen has been reported to be about 12 hours (26).

3.2 Embryo and tissue harvesting

Mouse embryos are staged from the time point when a vaginal plug was observed, which is designated as the stage E0.5. Embryos and neonate/adult mice are sacrificed, dissected and embryos/tissues washed in PBS before further processing. The respective national and local animal experimental guidelines need to be followed for the sacrifice of pregnant dams or newborn/adult animals.

3.3 Immunofluorescence analysis of reporter distribution on frozen sections

1. Embryos/tissues are fixed at room temperature in 4% freshly prepared paraformaldehyde (PFA) solution in PBS for 2–8 h (depending on the size) in 30-50 ml universal containers. The tissue is washed in PBS and cryoprotected by incubating in 15% sucrose solution at 4 °C until it sinks to the bottom of the container. This is followed by incubation in 30% sucrose solution at 4 °C until the tissue has sunk.

2. Embryos/tissues are placed in Peel-A-Ways or other embedding molds and as much of the sucrose solution is removed as possible using tissue paper before OCT is added to the mold to go at least 1 cm over the embryo/tissue. The embryos/tissues are snap frozen in liquid N_2-cooled isopentane. Plastic tubes with isopentane are kept for a few minutes in liquid N_2 and then the embryos are rapidly submerged in the isopentane.

Cooling of isopentane with dry ice is also possible. The frozen embryos are wrapped in foil and stored at -80°C.

3. Cryosections of 7-10 μm thickness are obtained on a cryostat, collected on glass slides and stored at -20 °C until use. YFP expression is strong enough to be directly detected by confocal microscopy (Figures 3A, B).

4. For immunofluorescence detection of reporter and marker proteins, cryosections are air-dried, rehydrated for 5 minutes in TPBS and blocked during 30 minutes for non-specific binding with SB or SBT for membrane-bound and intracellular antigens, respectively. When using biotinylated secondary antibodies, the endogenous biotin must be blocked at this step with the Avidin-Biotin blocking kit, according to the instructions on the supplier. In this case, the sections should be washed for 5 minutes in TPBS before adding the primary antibody.

5. Single immunofluorescence is performed incubating the sections with the primary antibody overnight at 4° C. The antibody is dissolved in SB or SBT as indicated in the former point. Then, the sections are washed in TPBS (3x5 minutes) and incubated with the corresponding fluorochrome-conjugated secondary antibody dissolved in SB or SBT for 1 hour at room temperature in the dark. If nuclei are to be counterstained with DAPI, the working solution can be added to the solvent of the secondary antibody to a final concentration of 1 μg/ml of DAPI. Alternatively, the sections can be stained with the working solution described in the section 2.3.9 (see Notes 7, 8).

6. For double immunofluorescence, two primary antibodies (for example a rabbit polyclonal and a mouse or rat monoclonal) are incubated in combination overnight

at 4°C, in SB or SBT as described in the former section. After a wash step with TPBS, the sections are incubated for 1 hour with the corresponding secondary antibodies conjugated to different fluorochromes (eg. phycoerythrin and Alexa-647) and DAPI (1 μg/mL final concentration), diluted in SB or SBT, allowing for three-color images (Figure 3A, B).

7. When the primary antibodies are generated in different species (e.g. rat, mouse and rabbit), it is even possible to perform triple immunofluorescence. In this case, rat and rabbit primary antibodies, and the corresponding secondary antibodies are incubated first as described above. Then, the sections are incubated for 1 hour at room temperature with monovalent donkey anti-mouse IgG, Fab fragment (see Note 9) diluted 1:100 in TPBS, followed by washing in TPBS (3x5 minutes) and incubation with the mouse primary antibody, washing and the corresponding anti-mouse IgG secondary antibody conjugated to a compatible fluorochrome with those used previously. Counterstaining with DAPI allows for four-color images, but it is important to take in account that confocal images are captured only in three channels (red, green and blue), being any additional color combination of them.

8. After final wash in TPBS (3x5 minutes), the sections are mounted with glycerol:PBS 1:1 or commercial mounting media and protected from the light.

3.4 X-gal staining on frozen sections

1. Embryos/tissues can be directly frozen in OCT without prior fixation, and stored at -80 °C until further use. Cryosections obtained from the unfixed tissue are fixed for 10 min in cold 4% PFA and washed three times in PBS.

2. The X-gal dilution buffer is set up, warmed to 37 °C and then 40 mg/ml X-gal stock solution added. The slides are incubated in X-gal working solution in a humidified chamber placed in an incubator at 37°C for 24 hours.

3. The sections are washed in PBS for 2 x 5 minutes (mins), rinse with distilled water briefly and counterstain with neutral red for 3-5 mins. After washing in distilled water the sections can be dehydrated in alcohol and mounted with Eukitt mounting medium.

See Note 10.

3.5 X-Gal whole mount staining of embryos/tissues

1. Large mouse embryos (over E13.5) or whole organs need to be sectioned with a scalpel (e.g. sagittally) to improve penetration of the staining solution. Embryos/tissues are fixed in 0.2% Glutaraldehyde/2% PFA fixative at 4 °C for 10-15 mins (small embryos), 15-30 mins (large embryos over E13.5) or 1-2 hours (adult tissues) in 20 ml universal containers.

2. The embryos/tissues are washed 3 times for 30 mins in wash solution, followed by incubation in X-gal staining solution in the dark at room temperature (see Note 11). The stained embryos/tissues are postfixed overnight in 4% PFA at 4 °C, and after a few PBS washes, stored in 70% ethanol.

3.6 Histological analysis of X-gal stained whole embryos/tissues

1. Postfixed X-gal stained embryos/tissues are dehydrated through an ethanol series

(25%, 50%, 75%) into 100% ethanol (twice), with each step lasting 15-30 mins at room temperature. The ethanol is then replaced by isopropanol for two incubation steps of at least 15 mins each.

2. The embryo/tissue is incubated in a pre-warmed, well-mixed 1:1 Isopropanol:Paraffin solution in a histology incubator at 58-60 °C for 1 hour. The Isopropanol:Paraffin solution is subsequently replaced by fresh paraffin and after a short incubation for 1 hour in the incubator, the embryo/tissue is incubated in a fresh change of paraffin in the incubator overnight.

3. The next day, the embryo/tissue is placed inside embedding molds or Peel-A-Ways and covered by paraffin. This step is easiest when done using an embedding station. It is important to consider the desired orientation of the sections and achieving the correct orientation especially for small embryos can be tricky (see Note 12). The tissue block is allowed to solidify overnight before sectioning. For long-term storage, the blocks are stored at 4 °C.

4. Using a microtome, sections of 5-10 μm are generated as 'ribbons', and collected on glass slides with the help of water (for example histology water bath at 45-50 °C) to facilitate even spreading of the sections. Sections are subsequently dried for 30 mins at RT, followed by baking in an incubator at 45-50 °C overnight. Sections can be stored in slide boxes at room temperature or 4 °C until further use (see Note 13).

5. For Eosin counter staining, sections are dewaxed by 2 incubation steps in Xylene (or Histoclear as alternative) in a glass rack in a staining dish on a stirrer, where the small stir bar is placed between glass rack and glass trough. Note that Xylene steps require work in a fume cupboard.

6. Pass the slides in the glass rack through a series of ethanol changes, starting with 2 changes of 100% Ethanol, and incubation steps in 95%, 75% and 50% Ethanol for 5 mins each. Slides are subsequently placed in PBS. It is important that sections must not be allowed to dry from this step onwards.

7. Slides are incubated for 1 min in Eosin staining solution (see Note 14, 15). This is followed by a wash step in tap water for 1 min, and a dehydration series from 50% to 100% Ethanol for 2-5 mins each. Slides are incubated twice for 2 mins in Xylene (or Histoclear) before embedding with mounting medium using coverslips. A typical result of this procedure is shown in Figure 8.

4. Notes

1. Note that Tamoxifen is an anti-cancer drug used in cancer patients. Tamoxifen acts as an antagonist of the estrogen receptor through its active metabolite 4-hydroxytamoxifen. It is therefore essential that health and safety aspects of workers exposed to Tamoxifen are being considered before work is started.
2. Different concentrations of Tamoxifen solutions, using different types of oils (peanut, sunflower), are reported in the literature.
3. Sucrose solutions will be prone to contamination with yeast or fungi. Discard container after 2 weeks.
4. For long term storage, upon opening the bottle, aliquot 25% Glutaraldehyde grade II solution into 10 ml aliquots (15 ml universal containers), and store at -20 °C.
5. Nonidet P-40 is highly viscous, so exact measurement of the 1 ml Nonidet P-40 may not be possible.

6. Tamoxifen can affect the delivery of litters in pregnant females. If lineage tracing is required to last from embryonic to postnatal stages, it is therefore recommended that the pups are recovered by Caesarian section and placed with a foster dam. Lineage tracing in newborn pups can be facilitated via Tamoxifen administration to the lactating dam.
7. Cryosections are routinely stained with a panel of antibodies to identify the (developmental) fate of the Wt1-expressing cell lineage. Use of secondary antibodies conjugated to fluorochromes compatible with YFP, such as TRITC, phycoerythrin, Cy5, Alexa-647, or infrared-emitting fluorochromes allows for triple or quadruple staining.
8. Negative controls should always be performed by incubating with non-immune species-matched isotype IgG instead of the primary antibodies.
9. The monovalent antibody blocks potential binding sites on the rat IgG for the anti-mouse IgG secondary antibody.
10. Alternatively, β-galactosidase expression can be detected by immunofluorescence (protocol 3.3) using the rabbit anti-β-galactosidase primary antibody (#559762) from Cappel/MPI (Figure 6).
11. Depending on the level of β-galactosidase expression, staining may take between a few hours to overnight (Figure 7).
12. Use fine forceps that are pre-warmed in the histology incubator or the embedding station, to orient the embryo in the paraffin, and place the mold onto the hot spot of the embedding station. This allows the paraffin to stay liquid for a bit longer. One of the problems can be the formation of a solid sheet at the surface, which prevents visualisation of the embryo. Removing the solidifying sheet at the surface repeatedly will provide an additional short time window in

which to orient the embryo. In general, it is important to work fast, and to know beforehand which way the embryo should be oriented.

13. X-gal staining on sections can be detected by eye or under a microscope (depending on the strength of the signal), allowing the selection of relevant sections for Eosin counter staining. It is important to note that the tissue in the paraffin sections does not allow for a proper evaluation unless dewaxed, counter-stained (optional), appropriately dehydrated and permanently mounted with cover slips.
14. The Eosin counter staining provides a pink staining to the tissue section. The Eosin staining solution is acidic and the staining reaction is based on its binding to basic cells and tissue structures, including the cytoplasm of cells.
15. The optimal time for incubation in the Eosin staining solution depends on its strength and needs to be determined individually.

5. References

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Figure Legends

Figure 1. Direct expression of GFP as a reporter for Wt1, in the $Wt1^{tm1Nhsn}$ mouse line, generated by replacing exon1 and part of the intronic region of the Wt1 gene by a GFP-cassette containing construct (Ref. 98). **A**. Wt1-GFP expression in glomeruli of an adult kidney bisected shortly after dissection and visualised with a fluorescence dissecting microscope. **B**. In this section of the kidney of an adult mouse, GFP was immunostained using the chicken polyclonal anti-GFP (Abcam, ab13970). Wt1 (GFP) expression is clearly localized in the glomerular podocytes. Endothelial cells are immunostained in red.

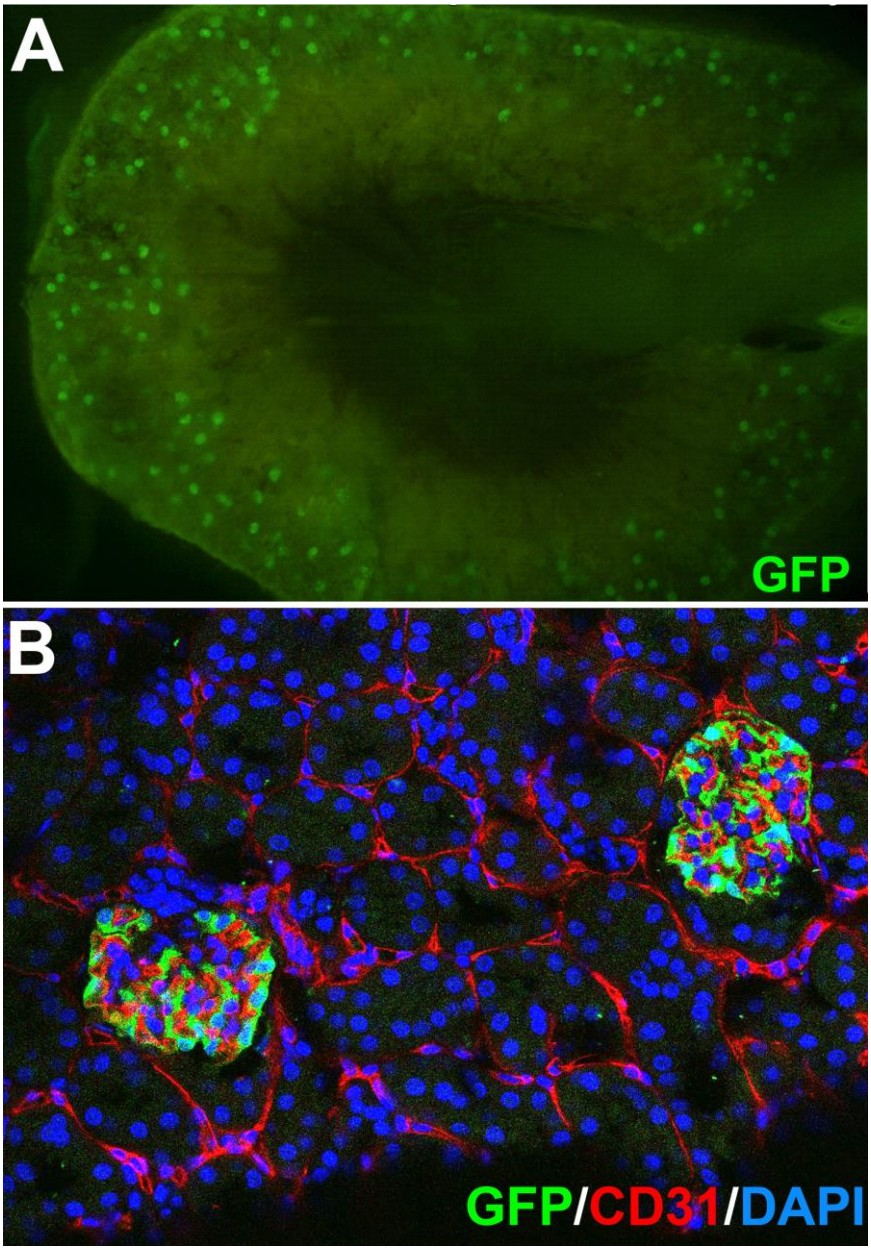

Figure 2. Schematic diagram delineating the constitutive Cre/loxP-based Wt1 lineage tracing system. The system consists of two genetic components: Transgenic mice generated by inserting a Cre recombinase expressing sequence under control of a Wt1 promoter ($Wt1^{Cre}$), and gene-targeted mice where a stop sequence flanked by lox sites is placed in front of a YPF cassette in the Rosa26 locus (Rosa26R-YFP reporter). Offspring of crosses between $Wt1^{Cre}$ and Rosa26-YFP reporter mice, result in embryos which carry both genetic modifications, allowing permanent expression of YFP in Wt1-expressing cells and their lineage. Thus, YFP expression remains active even when Wt1 and Cre recombinase expression has been switched off in a particular cell.

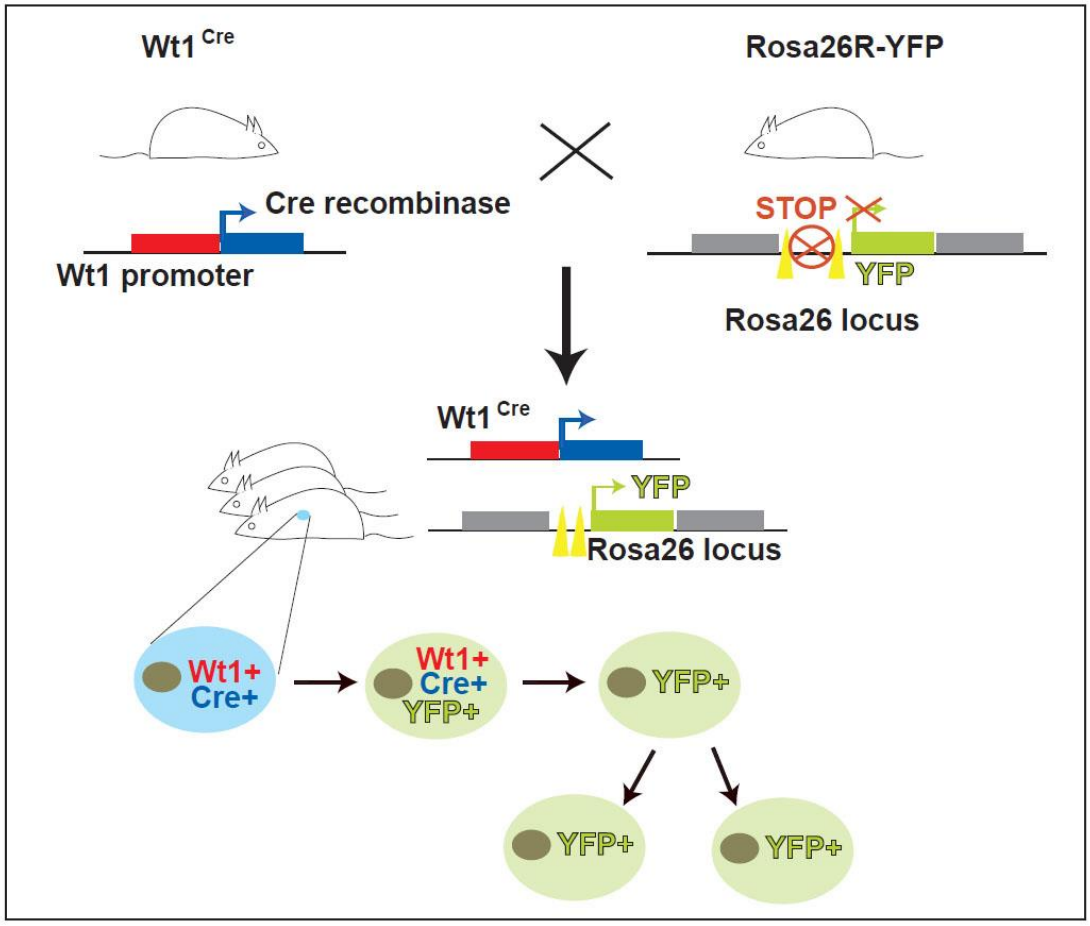

Figure 3. Permanent lineage tracing with the mWt1/IRES/GFP-Cre;Rosa26R-YFP model. The lineage of the cells that have expressed Wt1, constitutively express YFP which can be directly observed by confocal microscopy. **A.** Immunostaining of anoctamin (red) in the intestine of an E16.5 embryo. The calcium-activated chloride channel anoctamin is expressed in progenitors of the visceral smooth muscle, and some of which express YFP and therefore are part of the Wt1 lineage. **B.** Double immunofluorescence of an artery in the lungs of an E18.5 embryo. Smooth muscle is shown in red and endothelium in blue. YFP+ cells can be seen forming part of the endothelium (arrows). **C, D.** Despite the presence of a GFP sequence in the plasmid construct used to generate this model, expression of GFP is too low to be detected (C), and does not interfere with the strong expression of YFP (D).

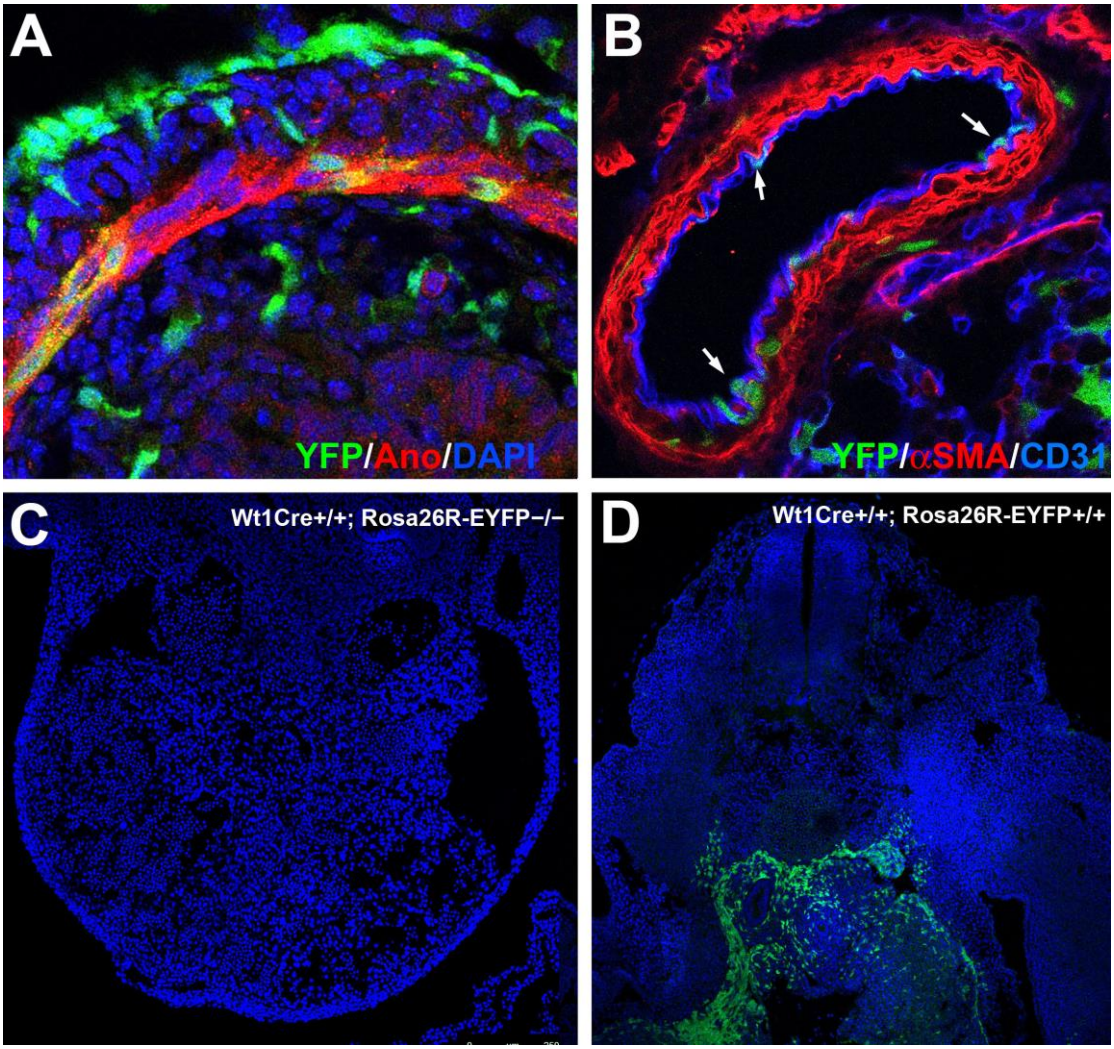

Figure 4. For temporal control of reporter expression (here: β-Galactosidase) in cells of the Wt1 lineage, the system consists of two genetic components similar to the model shown in Figure 2. However, to allow temporal control of Cre activity in Wt1-expressing cells, the Cre recombinase is fused to a modified estrogen receptor (ERT2; $Wt1^{CreERT2}$ mouse line). The Cre-ERT2 fusion protein can only enter the nucleus when binding of Tamoxifen to the ERT2 component of the fusion protein has led to a conformational change. In the absence of Tamoxifen, the Cre-ERT2 fusion protein is localised in the cytoplasm. In Tamoxifen-treated mice carrying both the $Wt1^{CreERT2}$ and the Rosa26R-LacZ reporter, the Cre recombinase will remove the loxP-flanked stop cassette after Tamoxifen administration leading to permanent reporter expression. However, cells expressing Wt1 in subsequent days will not be labelled through Cre recombination. This means that the lineage of Wt1 expressing cells can be followed from a specific, defined time point, for example during embryonic development.

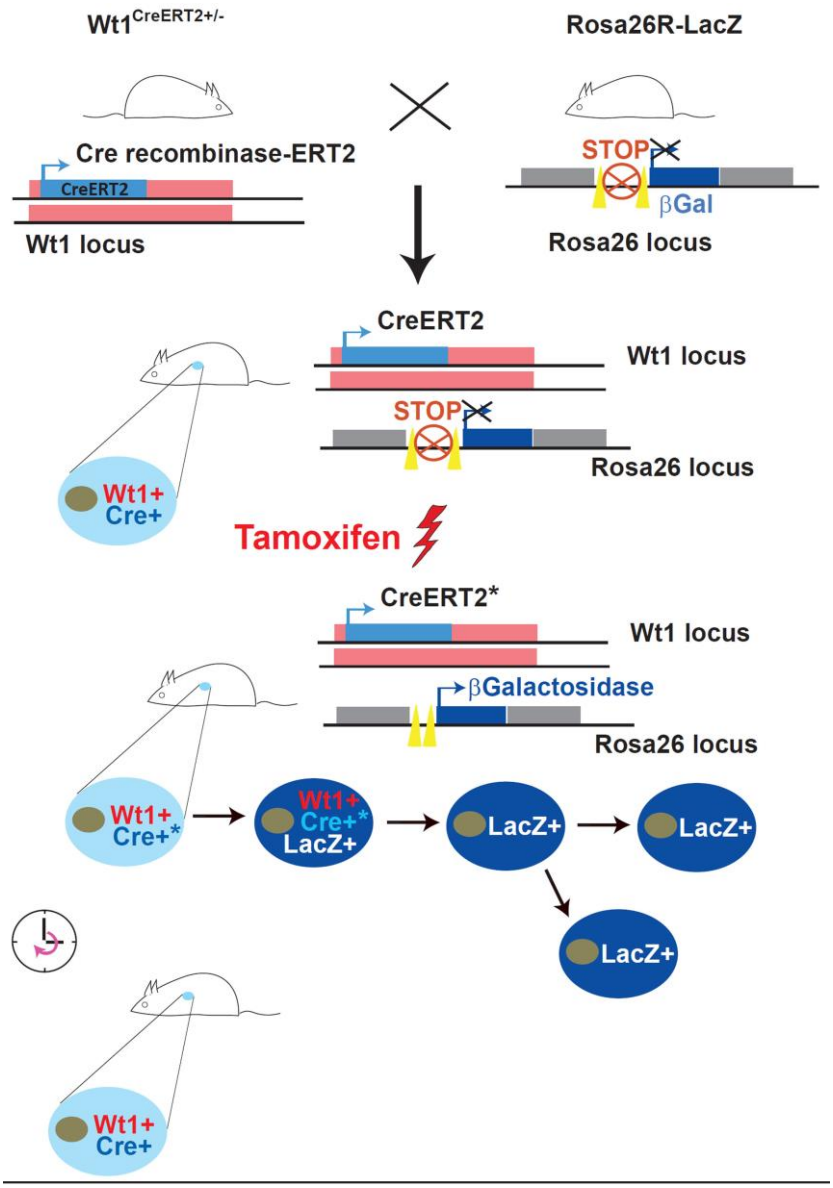

Figure 5. Endogenous GFP fluorescence of live mesothelial cells grown out of omentum explants in culture, isolated from a $Wt1^{CreERT2}$;Rosa26R-mTmG mouse 15 weeks after Tamoxifen-induced recombination. * indicate artificially blacked out areas of fluorescence of the omentum explant.

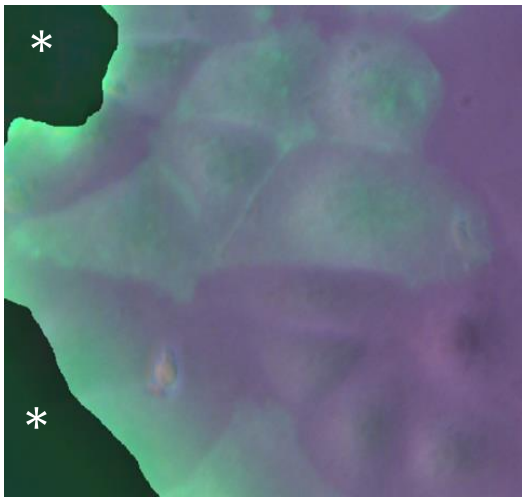

Figure 6. Detection of the β-Galactosidase reporter by immunofluorescence and confocal microscopy. **A.** Expression in an E13.5 embryo of the Tg(WT1)HNdh Wt1 reporter line. Putative Wt1 expressing cells can be seen in the mesothelium of the spleen primordium, located in the mesogastrium, and also in cells within the spleen. St, stomach. **B**. Immunolocalization of β-Galactosidase in the intestine of a E11.5 $Wt1^{Cre}$;Rosa26-LacZ embryo.

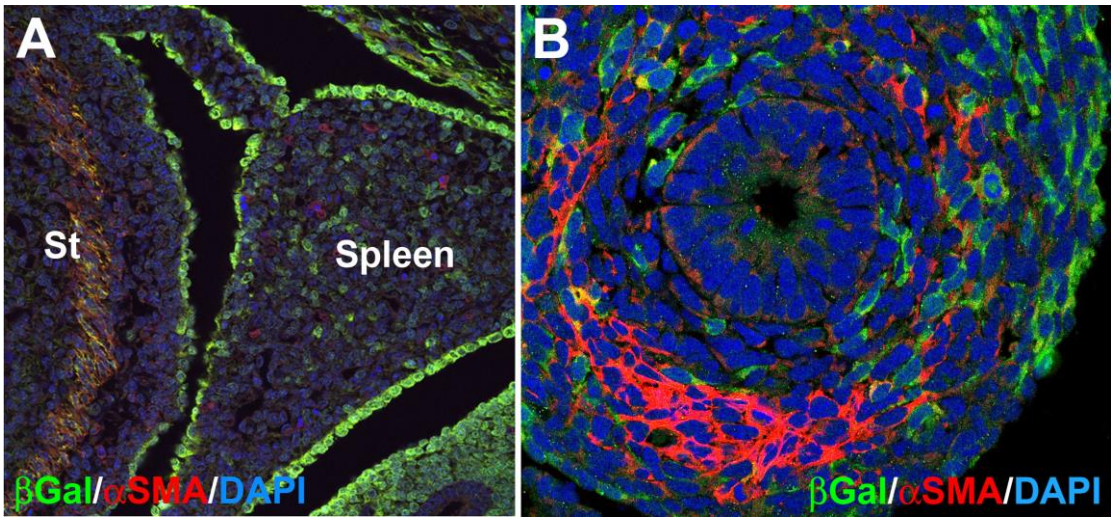

Figure 7. Detection of the β-Galactosidase reporter by X-gal staining of whole adult tissue. **A.** X-gal staining of bisected whole kidney of an adult $Wt1^{CreERT2}$;Rosa26R-LacZ mouse 5 months after Tamoxifen pulse. Only glomeruli show X-gal staining, indicating that they have expressed Wt1 at the time of Tamoxifen administration. **B.** Histological section of the X-gal-stained kidney shown in A, counterstained with Eosin. X-gal staining is confined to the glomeruli.

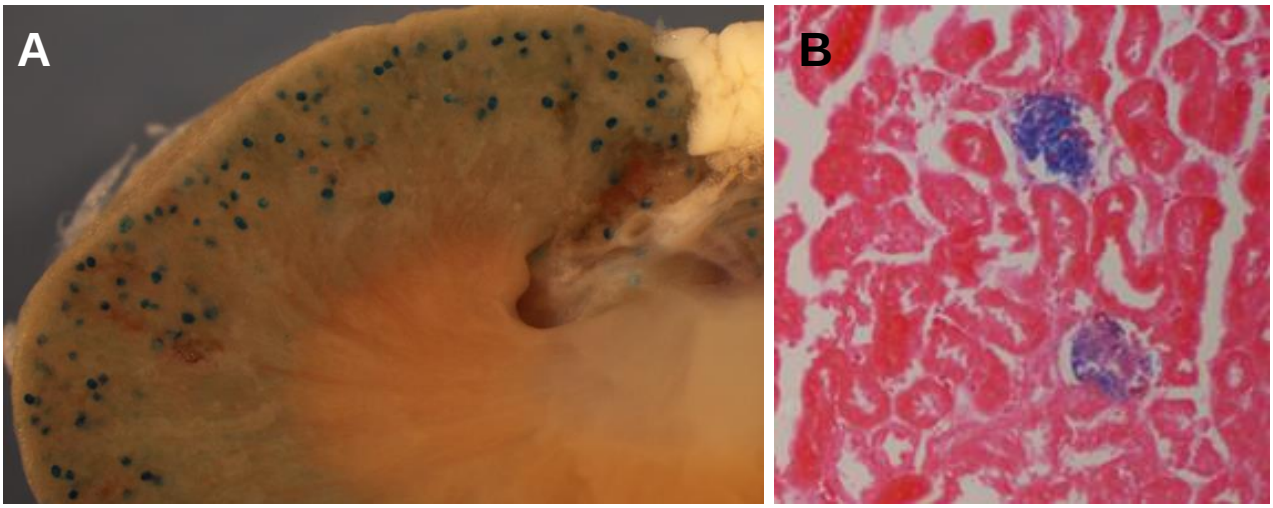

Figure 8. Detection of the β-Galactosidase reporter by X-gal staining of whole embryos which were stained, paraffin-embedded and sectioned. **A.** X-gal expression in an E10.5 embryo of the Tg(WT1)HNdh Wt1 reporter line, showing staining in intermediate mesoderm and mesenterium. **B.** X-gal expression staining cells from the Wt1 lineage in the digestive tract of an E11.5 mWt1/IRES/GFP-Cre;Rosa26LacZ embryo.