

AOP ID and Title:

SNAPSHOT

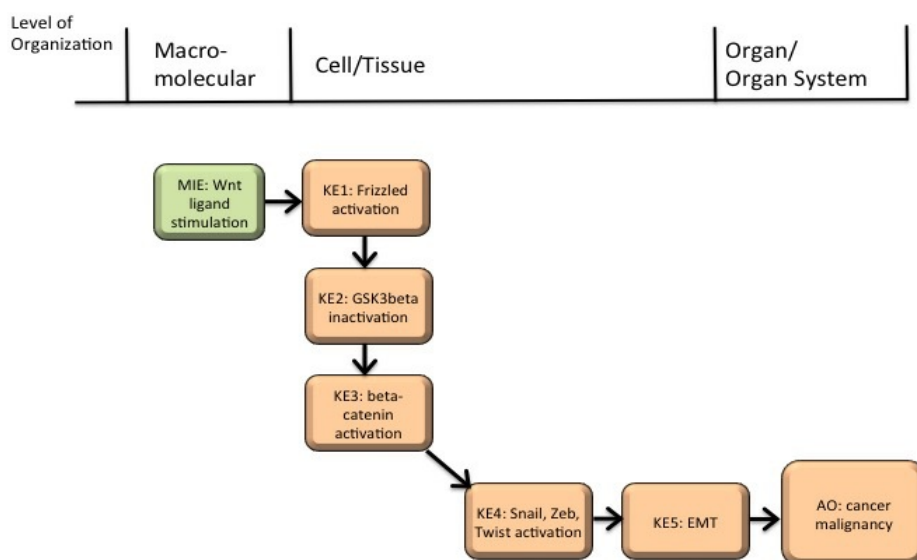
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AOP 298: Wnt ligand stimulation and Wnt signalling activation lead to cancer malignancy

Short Title: Wnt activation leading to cancer malignancy

Graphical Representation

AOP298 "Wnt ligand stimulation and Wnt signaling activation lead to cancer malignancy"



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Abstract

Wnt (Wingless and INT-1) ligands stimulate Frizzled receptors and activate Wnt signaling leading to cancer malignancy. This AOP workplan entitled "Wnt ligand stimulation and Wnt signaling activation lead to cancer malignancy" is suitable for the AOP programme in terms of revealing cancer signaling with the molecular signaling cascades induced by Wnt ligands. The current AOP includes MIE as Wnt ligand stimulation, KE1 as Frizzled activation, KE2 as GSK3beta inactivation, KE3 as beta-catenin activation, KE4 as Snail, Zeb, Twist1 activation, KE5 as epithelial-mesenchymal transition (EMT) and AO as cancer malignancy. The current AOP would be associated to the prediction of the cancer malignancy, which would be the regulatory toxicological

endpoint, by chemicals or molecules activating Wnt signaling. The Wnt/beta-catenin signaling is well understood in terms of development and cancer. The relationship between EMT and cancer malignancy has recently been investigated in many research fields such as molecular signatures of cells.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	1645	Wnt ligand stimulation (https://aopwiki.org/events/1645)	Wnt ligand stimulation
	KE	1646	Frizzled activation (https://aopwiki.org/events/1646)	Frizzled activation
	KE	1647	GSK3beta inactivation (https://aopwiki.org/events/1647)	GSK3beta inactivation
	KE	1648	β -catenin activation (https://aopwiki.org/events/1648)	β -catenin activation
	KE	1649	Snail, Zeb, Twist activation (https://aopwiki.org/events/1649)	Snail, Zeb, Twist activation
	KE	1650	Epithelial-mesenchymal transition (https://aopwiki.org/events/1650)	Epithelial-mesenchymal transition
	AO	1651	Cancer Malignancy (https://aopwiki.org/events/1651)	Cancer Malignancy

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Wnt ligand stimulation (https://aopwiki.org/relationships/1924)	adjacent	Frizzled activation	High	Moderate
Frizzled activation (https://aopwiki.org/relationships/1925)	adjacent	GSK3beta inactivation	High	Moderate
GSK3beta inactivation (https://aopwiki.org/relationships/1926)	adjacent	β -catenin activation	High	Moderate
β -catenin activation (https://aopwiki.org/relationships/1927)	adjacent	Snail, Zeb, Twist activation	High	Moderate
Snail, Zeb, Twist activation (https://aopwiki.org/relationships/1928)	adjacent	Epithelial-mesenchymal transition	High	Moderate
Epithelial-mesenchymal transition (https://aopwiki.org/relationships/1929)	adjacent	Cancer Malignancy	High	Low

Stressors

Name	Evidence
Wnt	High

Name	Evidence
WNT2	High

Wnt

WNT induces EMT (J. Zhang, Tian, & Xing, 2016).

WNT2

WNT2 induces EMT in cervical cancer (Zhou et al., 2016).

Overall Assessment of the AOP

1. Support for Biological Plausibility of KERs	
MIE => KE1: Wnt ligand stimulation leads to Frizzled activation	Biological Plausibility of the MIE => KE1 is high. Rationale: Upon the stimulation with Wnt ligand, Wnt ligand binds to FZD and form the complex with LRP5/6 (MacDonald et al., 2009).
KE1 => KE2: Frizzled activation leads to GSK3beta inactivation	Biological Plausibility of the KE1 => KE2 is high. Rationale: Upon Wnt ligand stimulation, FZD is activated and Axin is recruited to the phosphorylated tail of LRP dimerized with the activated FZD, the seven-transmembrane receptor, followed by GSK3beta inactivation to prevent beta-catenin degradation (Aberle, Bauer, Stappert, Kispert, & Kemler, 1997) (Clevers & Nusse, 2012).
KE2 => KE3: GSK3beta inactivation leads to β -catenin activation	Biological Plausibility of the KE2 => KE3 is high. Rationale: GSK3beta recruitment to LRP6 leads to form un-phosphorylated beta-catenin inducing the stabilization and translocation of the beta-catenin (MacDonald, Tamai, & He, 2009). Stabilized beta-catenin accumulates in cytosol and translocates into the nucleus leading to beta-catenin activation (MacDonald et al., 2009).

KE3 => KE4: β-catenin activation leads to Snail, Zeb, Twist activation	Biological Plausibility of the KE3 => KE4 is high. Rationale: The treatment of human gastric cancer cells with INC280, which inhibits c-MET overexpressed in diffuse-type gastric cancer with poor prognosis, shows downregulation in beta-catenin and Snail expression, (Sohn et al., 2019). The treatment with garcinol, a polyisoprenylated benzophenone derivative that is obtained from Garcinia indica extract, induced ZEB1 and ZEB2 down-regulation, increase in phosphorylated beta-catenin and decrease in nuclear beta-catenin in human breast cancer cells (Ahmad et al., 2012). Sortilin, a member of the Vps10p sorting receptor family which is highly expressed in high-grade malignant glioma, positively regulates GSK-3β/beta-catenin/Twist signaling pathway in glioblastoma (W. Yang et al., 2019).
KE4 => KE5: Snail, Zeb, Twist activation leads to Epithelial-mesenchymal transition, induced	Biological Plausibility of the KE4 => KE5 is high. Rationale: The transcription factors such as Snail, Zeb and Twist inhibit the CDH1 expression through their binding towards the promoter of CDH1, which leads to inhibition of cell adhesion and EMT (Diaz et al., 2014).

KE5 => AO: Epithelial-mesenchymal transition, induced leads to Cancer Malignancy	Biological Plausibility of the KE5 => AO is high. Rationale: The morphological and physiological changes associated with EMT are involved in invasiveness and drug resistance (Shibue & Weinberg, 2017). The EMT-activated particular carcinoma cells in primary tumors invade the surrounding stroma (Shibue & Weinberg, 2017). The EMT –activated carcinoma cells interact with the surrounding extracellular matrix protein to induce focal adhesion kinase and extracellular signal-related kinase activation, followed by the transforming growth factor beta (TGFbeta) and canonical and/or noncanonical Wnt pathways to induce cancer stem cell (CSC) properties which contribute to the drug resistance (Shibue & Weinberg, 2017). EMT-associated down-regulation of multiple apoptotic signaling pathways induce drug efflux and slow cell proliferation to induce the general resistance of carcinoma cells to anti-cancer drugs (Shibue & Weinberg, 2017). Snail, an EMT-related transcription factor, induces the expression of the AXL receptor tyrosine kinase, which enables the cancer cells to survive by the activation of AXL signaling triggered by the binding of its ligand growth arrest-specific protein 6 (GAS6)(Shibue & Weinberg, 2017). The EMT-activated cells evade the lethal effect of cytotoxic T cells, which include the elevated expression of programmed cell death 1 ligand (PD-L1) which binds to the programmed cell death protein 1 (PD-1) inhibitory immune-checkpoint receptor on the cell surface of cytotoxic T cells (Shibue & Weinberg, 2017).
2. Support for essentiality of KEs	
KE5: Epithelial-mesenchymal transition	Essentiality of the KE5 is moderate. Rationale for Essentiality of KEs in the AOP:
3. Empirical support for KERs	

MIE => KE1: Wnt ligand stimulation leads to Frizzled activation	Empirical Support of the MIE => KE1 is high. Rationale: Dishevelled (DVL), a positive regulator of Wnt signaling, forms the complex with FZD and leads to trigger the Wnt signaling together with Wnt coreceptor low-density lipoprotein (LDL) receptor-related protein 6 (LRP6) (Clevers & Nusse, 2012; X. Jiang et al., 2015). Wnt binds to FZD and activates the Wnt signaling (Clevers & Nusse, 2012; Janda et al., 2012; Nile et al., 2017). Wnt binding towards FZD induces the formation of the protein complex with LRP5/6 and DVL, leading to the down-stream signaling activation (Clevers & Nusse, 2012).
KE1 => KE2: Frizzled activation leads to GSK3beta inactivation	Empirical Support of the KE1 => KE2 is high. Rationale: The ligand-stimulated FZD induces the regulation of the phosphorylation by GSK3beta to inactivate GSK3beta which phosphorylates beta-catenin (Clevers & Nusse, 2012). The binding of Axin to the cytoplasmic tail of LRP5 bound to Wnt is crucial for the Wnt signaling pathway regulation and GSK3 beta inactivation in Wnt/beta-catenin signaling (Mao et al., 2001). Axin-LRP6 binding is the important step for the phosphorylation of the LRP5/6 tail by GSK3 beta which phosphorylates the serine in the PPPSP motif found in beta-catenin, Axin, APC (He, Semenov, Tamai, & Zeng, 2004; Tamai et al., 2004; Zeng et al., 2005). Wnt3a induces phosphorylation of LRP6 leading to beta-catenin activation, while beta-catenin is not activated in FZD-inhibited cells (Zeng et al., 2008).

KE2 => KE3: GSK3beta inactivation leads to β-catenin activation	Empirical Support of the KE2 => KE3 is high. Rationale: GSK3beta inactivation induces the beta-catenin stabilization (Pez et al., 2013). GSK3beta inactivation induces beta-catenin translocation into the nucleus (MacDonald et al., 2009; Pez et al., 2013). WNT2 knockdown induces the accumulation of GSK3beta in the cytoplasm and reduced the expression of beta-catenin, which WNT2 overexpression reduces the expression of GSK3beta in the cytoplasm and induces beta-catenin translocation into the nucleus (Wang, Li, & Kidder, 2010). WNT2 siRNA knockdown increases the GSK3beta expression and decreases beta-catenin expression, and WNT2 overexpression reduces the GSK3beta and increases beta-catenin in granulosa cells in Mus musculus (Wang et al., 2010).
KE3 => KE4: β-catenin activation leads to Snail, Zeb, Twist activation	Empirical Support of the KE3 => KE4 is high. Rationale: The inhibition of c-MET, which is overexpressed in diffuse-type gastric cancer, induced increase in phosphorylated beta-catenin, decrease in beta-catenin and Snail (Sohn et al., 2019). The garcinol, that has anti-cancer effect, increases phosphorylated beta-catenin, decreases beta-catenin and ZEB1/ZEB2, and inhibit EMT (Ahmad et al., 2012). The inhibition of sortilin by AF38469 (a sortilin inhibitor) or small interference RNA (siRNA) results in decrease in beta-catenin and Twist expression in human glioblastoma cells (W. Yang et al., 2019).
KE4 => KE5: Snail, Zeb, Twist activation leads to Epithelial- mesenchymal transition, induced	Empirical Support of the KE4 => KE5 is high. Rationale: Histone deacetylase inhibitors affect on EMT-related transcription factors including ZEB, Twist and Snail (Wawruszak et al., 2019). Snail and Zeb induces EMT and suppress E-cadherin (CDH1) (Batlle et al., 2000; Diaz et al., 2014; Peinado, Olmeda, & Cano, 2007).

KE5 => AO: Epithelial-mesenchymal transition, induced leads to Cancer Malignancy	Empirical Support of the KE5 => AO is high. Rationale: Slug/Snai2, a ces-1-related zinc finger transcription factor gene, confers resistance to p53-mediated apoptosis of hematopoietic progenitors by repressing PUMA (also known as BBC3, encoding Bcl-2-binding component 3) (Inukai et al., 1999; Shibue & Weinberg, 2017; W.-S. Wu et al., 2005). EMT activation induces the expression of multiple members of the ATP-binding cassette (ABC) transporter family, which results in the resistant to doxorubicin (Saxena, Stephens, Pathak, & Rangarajan, 2011; Shibue & Weinberg, 2017) TGFbeta-1 induced EMT results in the acquisition of cancer stem cell (CSC) like properties (Pirozzi et al., 2011; Shibue & Weinberg, 2017). Snail-induced EMT induces the cancer metastasis and resistance to dendritic cell-mediated immunotherapy (Kudo-Saito, Shirako, Takeuchi, & Kawakami, 2009). Zinc finger E-box-binding homeobox (ZEB1)-induced EMT results in the relief of miR-200-mediated repression of programmed cell death 1 ligand (PD-L1) expression, a major inhibitory ligand for the programmed cell death protein (PD-1) immune-checkpoint protein on CD8+ cytotoxic T lymphocyte (CTL), subsequently the CD8+ T cell immunosuppression and metastasis (Chen et al., 2014).
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Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Sex Applicability

Sex	Evidence
Unspecific	High

Homo sapiens

Essentiality of the Key Events

Wnt signaling is involved in cancer malignancy (Tanabe, 2018).

Key Events Frizzled activation, GSK3beta, beta-catenin activation and Zeb, Twist and Snail transcription factors are essential to this AOP.

Upon stimulation with Wnt ligand to Frizzled receptor, Wnt/beta-catenin signaling is activated. Wnt/beta-catenin consists of GSK3 beta inactivation, beta-catenin activation and up-regulation of transcription factors such as Zeb, Twist and Snail. The transcription factors Zeb, Twist

and Snail relate to the activation of EMT-related genes. EMT is regulated with various gene networks (S. Tanabe, 2015).

Weight of Evidence Summary

The Wnt signaling promotes EMT and cancer malignancy in colorectal cancer (Lazarova & Bordonaro, 2017). Although the potential pathways other than Wnt signaling exist in EMT induction and the mechanism underlain cancer malignancy, Wnt signaling is one of the main pathways to induce EMT and cancer malignancy (Polakis, 2012).

Quantitative Consideration

Wnt signaling activates the CSCs to promote cancer malignancy (Reya & Clevers, 2005). The responses between each KEs from Frizzled activation and GSK3 β inactivation, beta-catenin activation, Snail, Zeb, Twist activation are dose-dependently related. The quantification of EMT and cancer malignancy would require the further investigation.

Considerations for Potential Applications of the AOP (optional)

AOP entitled “Wnt ligand stimulation and Wnt signaling activation lead to cancer malignancy” might be utilized for the development and risk assessment of anti-cancer drugs. EMT is involved in the acquisition of drug resistance, which is one of the critical features of cancer malignancy. The assessment of EMT would be the potential prediction of the adverse effects of anti-cancer drugs.

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Appendix 1

List of MIEs in this AOP

Event: 1645: Wnt ligand stimulation (<https://aopwiki.org/events/1645>)

Short Name: Wnt ligand stimulation

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:298 - Wnt ligand stimulation and Wnt signalling activation lead to cancer malignancy (https://aopwiki.org/aops/298)	MolecularInitiatingEvent

Stressors

Name
WNT2
Wnt
WNT5A

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
cell

Organ term

Organ term
organ

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

•WNT secretion from the cells requires a number of factors including the sorting receptor Wntless (Banziger et al., 2006; Bartscherer, Pelte, Ingelfinger, & Boutros, 2006; Goodman et al., 2006).

Wnt signaling activation is occurred by WNTs. WNT5A can activate Wnt signaling leading to EMT (Dissanayake et al., 2007) and inhibit Wnt signaling as well (Kremenevskaja et al., 2005).

Dickkopf WNT signaling pathway inhibitor 2 (DKK2) inhibits Wnt signaling (Mu et al., 2017).

Zebrafish eaf1 and eaf2/u19 mediate effective convergence and extension movements through the maintenance of wnt11 and wnt5 expression (J. X. Liu, Hu, Wang, Gui, & Xiao, 2009).

The inhibitors for WNT signaling include WNT inhibitory factor 1 (WIF-1) (Yan et al., 2018; Jiao Zhang et al., 2014).

The casein kinase 1 (CK1), serine (Ser)/threonine (Thr) protein kinases are involved in Wnt and Hedgehog pathways (J. Jiang, 2017).

IQ-1 and ID-8 are modulators for Wnt signaling (Hasegawa et al., 2012; Miyabayashi et al., 2007) (Kahn, 2014).

WNT activates Wnt signaling which is involved in neuroblastoma (Johnsen, Dyberg, Fransson, & Wickström, 2018).

Wnt5a is involved in non-canonical WNT signaling pathways, through binding to different members of the Frizzled- and Ror-family receptors (Asem, Buechler, Wates, Miller, & Stack, 2016).

WNT5A induces EMT via non-canonical WNT signaling related to molecules such as PKC and JNK (Dissanayake et al., 2007; Jordan et al., 2013)) Abell et al., 2011).

WNT2B/WNT13 has been identified as human genes, and induces mesenchymal to epithelial transition (MET) in cooperation with FZD in colorectal cancer (Schwab et al., 2018).

Wnt8 activates WNT/beta-catenin signaling leading to induction of EMT in Sea urchin (Thiery, Acloque, Huang, & Nieto, 2009).

WNT2

WNT2 induces EMT in cervical cancer (Zhou et al., 2016).

Wnt

Wnt stimulation induces EMT.

WNT5A

WNT5A induces EMT in non-small-cell lung cancer (Wang, Tang, Gong, Zhu, & Liu, 2017).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

- The up-regulation of WNT ligand expression occurs in *Homo sapiens* (B. Wang et al., 2017).
- The Wnt genes play an important roles in the secretion from cells, glycosylation and tight association with the cell surface and extracellular matrix in *Drosophila melanogaster* (Willert & Nusse, 2012).
- Wnt5a expression leads to epithelial-mesenchymal transition (EMT) and metastasis in non-small-cell lung cancer in *Homo sapiens* (B. Wang et al., 2017).
- WNT2 expression lead to EMT induction in *Homo sapiens* (Zhou et al., 2016).

Key Event Description

Site of action: The site of action for the molecular initiating event is the cell membrane.

WNTs are secreted proteins that contain 22-24 conserved cysteine residues (Foulquier et al., 2018). The WNT molecules consist of molecular families including WNT1, WNT2, WNT2B/WNT13, WNT3, WNT4, WNT5A, WNT5B, WNT6, WNT7A, WNT7B, WNT8A, WNT8B, WNT10B, WNT11 and WNT16. (Clevers & Nusse, 2012; Katoh, 2001; Kusserow et al., 2005)

Wnt proteins consists of 350-400 amino acids (Saito-Diaz et al., 2013).

WNT ligands are known to trigger at least three different downstream signaling cascades including canonical WNT/beta-catenin signaling pathway, non-canonical WNT/Ca²⁺ pathway and planar cell polarity (PCP) pathway (De, 2011; Lai, Chien, & Moon, 2009; Willert & Nusse, 2012). WNTs bind to Frizzled proteins, which are seven-pass transmembrane receptors with an extracellular N-terminal cysteine-rich domain (Bhanot et al., 1996; Clevers, 2006). Wnt signaling begins with the binding of Wnt ligand towards the Frizzled receptors (Mohammed et al., 2016).

Canonical Wnt pathway consists of Wnt, GSK3beta and beta-catenin cascade (Clevers & Nusse, 2012; Hatsell, Rowlands, Hiremath, & Cowin, 2003).

How it is Measured or Detected

- Secretion of WNT requires a number of other dedicated factors including the sortin receptor Wntless (WLS), which binds to Wnt and escorts it to the cell surface (Banziger et al., 2006; Ching & Nusse, 2006)
- Wnt signaling is activated by the gene mutations of the signaling components (Ziv et al., 2017).
- Wnt1, Wnt3a and Wnt5a protein expression are measured by immunoblotting using antibodies for Wnt1, Wnt3a and Wnt5a, respectively (J. Du et al., 2016; B. Wang et al., 2017).
- WNT2, of which expression is detected by quantitative PCR, immunoblotting and immunohistochemistry, induces EMT (Zhou et al., 2016).
- Wnt2B (Wnt13) mediates mesenchymal-epithelial-transition (MET) *in vitro* (*Homo sapiens*) (Schwab et al., 2018).

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List of Key Events in the AOP

Event: 1646: Frizzled activation (<https://aopwiki.org/events/1646>)

Short Name: Frizzled activation

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:298 - Wnt ligand stimulation and Wnt signalling activation lead to cancer malignancy (https://aopwiki.org/aops/298)	KeyEvent

Stressors

Name
Wnt ligand

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
cell

Organ term

Organ term
organ

Evidence for Perturbation by Stressor

Wnt ligand

Wnt ligands bind to Frizzled receptor leading to the activation (Bhanot et al., 1996; Janda, Waghray, Levin, Thomas, & Garcia, 2012).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Oligomerization of FZD and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) activates Wnt/beta-catenin signaling in *Homo sapiens*. (Hua et al., 2018).

Key Event Description

Wnt ligands bind to Frizzled (FZD) receptors which are seven transmembrane-domain protein receptors (Nile, Mukund, Stanger, Wang, & Hannoush, 2017). At least 10 FZD receptors are identified in human cells. FZD receptor is activated by Wnt ligand binding (MacDonald, Tamai, & He, 2009).

Dishevelled (DVL), a positive regulator of Wnt signaling, form the complex with FZD and lead to trigger the Wnt signaling together with Wnt coreceptor low-density lipoprotein (LDL) receptor-related protein 6 (LRP6) (Clevers & Nusse, 2012; X. Jiang, Charlat, Zamponi, Yang, & Cong, 2015). DVL, however, has a controversial role to promote Wnt receptor degradation (X. Jiang et al., 2015). Meanwhile, DVL-dependent regulation of FZD level is involved in mTORC1 signaling suppression via Wnt/beta-catenin signaling (H. Zeng et al., 2018).

How it is Measured or Detected

- Frizzled receptor protein level on the cell surface is measured by flow cytometry with pan-FZD antibody (X. Jiang et al., 2015; H. Zeng et al., 2018).
- DVL protein level is measured by immunoblotting with anti-DVL2 antibody (H. Zeng et al., 2018).
- Fzd mRNA level is measured by quantitative reverse transcription-polymerase chain reaction (RT-PCR) (H. Zeng et al., 2018).

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Event: 1647: GSK3beta inactivation (<https://aopwiki.org/events/1647>)

Short Name: GSK3beta inactivation

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:298 - Wnt ligand stimulation and Wnt signalling activation lead to cancer malignancy (https://aopwiki.org/aops/298)	KeyEvent

Stressors

Name
CHIR99021
BIO (6-bromoindirubin-3'-oxime)
Kenpaullone
SB216763
TWS119
CHIR98014

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
cell

Organ term

Organ term
organ

Evidence for Perturbation by Stressor

CHIR99021

CHIR99021 inhibits GSK3beta (Wu et al., 2015) .

BIO (6-bromoindirubin-3'-oxime)

BIO (6-bromoindirubin-3'-oxime) inhibits GSK3beta (Wu et al., 2015).

Kenpaullone

Kenpaullone inhibits GSK3beta (Yang et al., 2013).

SB216763

SB216763 inhibits GSK3betat (Naujok, Lentjes, Diekmann, Davenport, & Lenzen, 2014).

TWS119

TWS119 inhibits GSK3beta (Tang et al., 2018).

CHIR98014

CHIR98014 inhibits GSK3beta (Guerrero et al., 2014; Lian et al., 2014).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Phosphorylation of GSK3beta is induced, which means the inactivation of GSK3beta, in *Homo sapiens* (Huang et al., 2019).

Key Event Description

Glycogen synthase kinase 3beta (GSK3 beta) is inhibited by CHIR99021 (C. H. Li et al., 2017; C. C. Liu et al., 2016; Sineva & Pospelov, 2010).

Glycogen synthase kinase 3beta (GSK3 beta) is inhibited by BIO (6-bromoindirubin-3'-oxime) (Mohammed et al., 2016; Sineva & Pospelov, 2010).

Kenpaullone is a dual inhibitor for GSK3 α/β and HPK1/GCK-like kinase (Y. M. Yang et al., 2013; Yao et al., 1999).

CHIR and BIO treatments lead to a slight upregulation of the primary transcripts of the miR-302-367 cluster and miR-181 family of miRNAs, which activate Wnt/ β -catenin signaling (Y. Wu et al., 2015).

SB216763 inhibits GSK3 β (Naujok et al., 2014).

TWS119 inhibits GSK3 β (Tang et al., 2018).

CHIR99014 inhibits GSK3 β (Guerrero et al., 2014; Lian et al., 2014).

How it is Measured or Detected

Inactivation of GSK3 β is measured by Wnt/ β -catenin activity assay, in which the vector containing the firefly luciferase gene controlled by TCF/LEF binding sites is transfected in the cells (Naujok et al., 2014). Phosphorylation of GSK3 β at residue Ser9 leads to the inactivation of GSK3 β . Phosphorylation of GSK3 β is measured by immunoblotting with anti-phospho-GSK3 β (Huang et al., 2019).

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Event: 1648: β -catenin activation (<https://aopwiki.org/events/1648>)

AOP298

Short Name: β -catenin activation

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:298 - Wnt ligand stimulation and Wnt signalling activation lead to cancer malignancy (https://aopwiki.org/aops/298)	KeyEvent

Stressors

Name
CHIR-99021
CHIR-98014

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
cell

Organ term

Organ term
organ

Evidence for Perturbation by Stressor

CHIR-99021

CHIR-99021 activates beta-catenin (Naujok et al., 2014).

CHIR-98014

CHIR-98014 activates beta-catenin (Naujok et al., 2014).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

- Beta-catenin is stabilized and translocated into nucleus in *Homo sapiens* (Huang et al., 2019).
- Beta-catenin is activated in *Homo sapiens* (Huang et al., 2019) (Naujok et al., 2014).

Key Event Description

Upon the Wnt signaling activation, beta-catenin is stabilized and activated via inhibition of the phosphorylation by GSK3beta (Huang et al., 2019). Once the beta-catenin is stabilized, it translocates into the nucleus and enhance the expression of target genes of Wnt/beta-catenin signaling pathway (Huang et al., 2019). Beta-catenin activation is related to cancer(Tanabe, 2014).

How it is Measured or Detected

- The beta-catenin level in nucleus is measured by immunoblotting with anti-beta-catenin antibody (Huang et al., 2019)
- The beta-catenin nuclear translocation is measured by immunofluorescence assay (Huang et al., 2019).
- Activity of beta-catenin is measured by Wnt/beta-catenin activity assay, in which the vector containing the firefly luciferase gene controlled by TCF/LEF binding sites is transfected in the cells (Naujok et al., 2014).

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Event: 1649: Snail, Zeb, Twist activation (<https://aopwiki.org/events/1649>)

Short Name: Snail, Zeb, Twist activation

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:298 - Wnt ligand stimulation and Wnt signalling activation lead to cancer malignancy (https://aopwiki.org/aops/298)	KeyEvent

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
cell

Organ term

Organ term
organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

- Snail and Twist transcription factor is up-regulated in *Homo sapiens* (Huang et al., 2019).
- ZEB1 promoter activity is increased in *Homo sapiens* (Kwon et al., 2016).
- Twist1 is activated in *Mus musculus* (Laursen, Mielke, Iannaccone, & Fuchtbauer, 2007).

Key Event Description

- ZEB is one of the important transcription factors for EMT regulation (Zhang, Sun, & Ma, 2015).
- SNAI1 (Snail) is an important transcription factor for cell differentiation and survival, and the phosphorylation and nuclear localization of Snail1 induced by Wnt signaling pathways are critical for the regulation of EMT (Kaufhold & Bonavida, 2014).
- Transcription factors SNAI1 and TWIST1 induce EMT (Hodge, Cui, Gamble, & Guo, 2018) (Mani et al., 2008)
- It is suggested that Sp1, a transcription factor involved in cell growth and metastasis, is induced by cytochrome P450 1B1 (CYP1B1), and promotes EMT, which leads to cell proliferation and metastasis (Kwon et al., 2016).

How it is Measured or Detected

- The activation of Snail and Twist are measured with gene expression up-regulation by real-time PCR (Huang et al., 2019).
- ZEB1 and TWIST1 activation is measured by dual luciferase reporter assay detecting the promoter activation (Kwon et al., 2016)
- The protein expression of ZEB2, SNAI1 are measure by western blotting with antibodies (Kwon et al., 2016).

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Event: 1650: Epithelial-mesenchymal transition (<https://aopwiki.org/events/1650>)

Short Name: Epithelial-mesenchymal transition

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:298 - Wnt ligand stimulation and Wnt signalling activation lead to cancer malignancy (https://aopwiki.org/aops/298)	KeyEvent

Stressors

Name
GOLPH3
LiCl
D-2-hydroxyglutarate

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
cell

Organ term

Organ term
organ

Evidence for Perturbation by Stressor

GOLPH3

GOLPH3 induces EMT (Sun et al., 2017).

LiCl

LiCl induces EMT (Fang et al., 2018).

D-2-hydroxyglutarate

D-2-hydroxyglutarate induces EMT (Colvin et al., 2016).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

EMT is induced in cancer and involved in cancer metastasis in *Homo sapiens* (Suarez-Carmona, Lesage, Cataldo, & Gilles, 2017) (Du & Shim, 2016).

Key Event Description

- Epithelial-mesenchymal transition (EMT) is a phenomenon in which the cells transit from epithelial-like into mesenchymal-like phenotypes (S. Tanabe, 2017; Shihori Tanabe, Komatsu, Kazuhiko, Yokozaki, & Sasaki, 2015). In cancer, cells exhibiting EMT features contribute into the metastasis and drug resistance.
- It is known that D-2-hydroxyglutarate induces EMT (Guerra et al., 2017; Jia, Park, Jung, Levine, & Kaiparettu, 2018; Mishra et al., 2018; Sciacovelli & Frezza, 2017). D-2-hydroxyglutarate, an inhibitor of Jumonji-family histone demethylase, increased the trimethylation of histone H3 lysine 4 (H3K4) in the promoter region of the ZEB1, followed by the induction of EMT (Colvin et al., 2016).
- Wnt5a induces EMT and metastasis in non-small-cell lung cancer (Wang, Tang, Gong, Zhu, & Liu, 2017).
- EMT is related to Wnt/beta-catenin signaling and important for cancer (S. Tanabe, Kawabata, Aoyagi, Yokozaki, & Sasaki, 2016)
- TGFbeta induces EMT (Wendt, Smith, & Schiemann, 2010).

How it is Measured or Detected

- TGFbeta induces EMT *in vitro* (Willis et al., 2005).
- EMT can be detected by immunostaining with pro-surfactant protein-C (pro-SPC) and N-cadherin in idiopathic pulmonary fibrosis (IPF) lung *in vivo* (Kim et al., 2006).
- TGFbeta induces EMT, which can be detected by immunostaining with vimentin in lung aloevela *in vivo* (Kim et al., 2006).

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List of Adverse Outcomes in this AOP

Event: 1651: Cancer Malignancy (<https://aopwiki.org/events/1651>)

Short Name: Cancer Malignancy

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:298 - Wnt ligand stimulation and Wnt signalling activation lead to cancer malignancy (https://aopwiki.org/aops/298)	AdverseOutcome

Biological Context

Level of Biological Organization
Tissue

Organ term

Organ term
organ

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Cancer malignancy such as drug resistance occurs in *Homo sapiens* (Du & Shim, 2016).

Key Event Description

- Cancer malignancy such as drug resistance is involved in EMT, which is an important phenomenon exhibiting the feature similar to cancer stem cells (CSCs) (Du & Shim, 2016).
- EMT is involved in metastasis and therapy resistance (Smith & Bhowmick, 2016).
- Diffuse-type gastric cancer which has a poor prognosis may be related to EMT (Tanabe, Aoyagi, Yokozaki, & Sasaki, 2014).

How it is Measured or Detected

Cancer malignancy and EMT can be detected with biomarkers (Zeisberg & Neilson, 2009).

Regulatory Significance of the AO

Cancer malignancy is very important in the cancer treatment, since the cancer metastasis and recurrence are one of the main obstacles to cure cancer.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 1924: Wnt ligand stimulation leads to Frizzled activation (<https://aopwiki.org/relationships/1924>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Wnt ligand stimulation and Wnt signalling activation lead to cancer malignancy (https://aopwiki.org/aops/298)	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

Wnt ligand stimulation leads to FZD activation in *Homo sapiens* (Clevers & Nusse, 2012).

Key Event Relationship Description

Wnt ligand binds to Frizzled receptor (FZD), which leads to the Wnt signaling activation (Nile, Mukund, Stanger, Wang, & Hannoush, 2017).

Evidence Supporting this KER

Biological Plausibility

Upon the stimulation with Wnt ligand, Wnt ligand binds to FZD and form the complex with LRP5/6 (MacDonald et al., 2009).

Empirical Evidence

Dishevelled (DVL), a positive regulator of Wnt signaling, form the complex with FZD and lead to trigger the Wnt signaling together with Wnt coreceptor low-density lipoprotein (LDL) receptor-related protein 6 (LRP6) (Clevers & Nusse, 2012; X. Jiang et al., 2015).

Wnt binds to FZD and activate the Wnt signaling (Clevers & Nusse, 2012; Janda et al., 2012; Nile et al., 2017). Wnt binding towards FZD induce the formation of the protein complex with LRP5/6 and DVL, leading to the down-stream signaling activation (Clevers & Nusse, 2012).

Uncertainties and Inconsistencies

Some Wnt ligands bind to FZD, leading to Wnt/beta-catenin signaling inactivation. DVL, a positive regulator of Wnt signaling, has a controversial role to promote Wnt receptor degradation (X. Jiang et al., 2015). DVL-dependent regulation of FZD level is involved in mTORC1 signaling suppression via Wnt/beta-catenin signaling (H. Zeng et al., 2018).

Quantitative Understanding of the Linkage

Response-response relationship

FZD5 can activate WNT3A/beta-catenin signaling in a dose-dependent manner (Hua et al., 2018). The increase in FZD5 protein enhances cell response to WNT3A. (Hua et al., 2018). LRP5 can augment WNT3A/beta-catenin signaling in a dose-dependent manner (Hua et al., 2018).

Time-scale

FZD7 enhances the activity of canonical Wnt/beta-catenin signaling with the treatment of WNT3A for 1 to 6 hrs (Cao et al., 2017).

Known modulating factors

The binding of Wnt and FZD induce the formation of the protein complex with the Dvl, Axin, CK1 GSK3, beta-catenin and APC to induce the beta-catenin translocation into the nucleus (Clevers & Nusse, 2012).

Known Feedforward/Feedback loops influencing this KER

The Wnt ligand is antagonized with secreted Frizzled-related proteins (sFRPs) and Wnt inhibitory protein (WIF), both of which can bind Wnts and inhibit interactions between WNT and FZD (Bovolenta, Esteve, Ruiz, Cisneros, & Lopez-Rios, 2008; Clevers & Nusse, 2012).

The Dickkopf 1 (DKK1) can disrupts Wnt-induced FZD-LRP6 complex formation (Clevers & Nusse, 2012; Ellwanger et al., 2008; Semenov, Zhang, & He, 2008).

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Relationship: 1925: Frizzled activation leads to GSK3beta inactivation (<https://aopwiki.org/relationships/1925>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Wnt ligand stimulation and Wnt signalling activation lead to cancer malignancy (https://aopwiki.org/aops/298)	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship**Taxonomic Applicability**

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

FZD activation leads to GSK3beta inactivation by the sequestration inside multivesicular endosomes in *Homo sapiens* (Taelman et al., 2010).

Key Event Relationship Description

Frizzled receptor (FZD) activation leads to Glycogen synthase kinase 3 (GSK3) beta inactivation, which leads to dephosphorylation of beta-catenin (Clevers & Nusse, 2012).

Evidence Supporting this KER**Biological Plausibility**

Upon Wnt ligand stimulation, FZD is activated and Axin is recruited to the phosphorylated tail of LRP dimerized with the activated FZD, the seven-transmembrane receptor, followed by GSK3beta inactivation to prevent beta-catenin degradation (Aberle, Bauer, Stappert, Kispert, & Kemler, 1997) (Clevers & Nusse, 2012).

Empirical Evidence

- The ligand-stimulated FZD induces the regulation of the phosphorylation by GSK3beta to inactivate GSK3beta which phosphorylates beta-catenin (Clevers & Nusse, 2012).
- The binding of Axin to the cytoplasmic tail of LRP5 bound to Wnt is crucial for the Wnt signaling pathway regulation and GSK3 beta inactivation in Wnt/beta-catenin signaling (Mao et al., 2001).
- Axin-LRP6 binding is the important step for the phosphorylation of the LRP5/6 tail by GSK3 beta which phosphorylates the serine in the PPPSP motif found in beta-catenin, Axin, APC (He, Semenov, Tamai, & Zeng, 2004; Tamai et al., 2004; Zeng et al., 2005).

Wnt3a induces phosphorylation of LRP6 leading to beta-catenin activation, while beta-catenin is not activated in FZD-inhibited cells (Zeng et al., 2008).

Uncertainties and Inconsistencies

- WNT5A inhibits WNT/beta-catenin signaling and exhibits tumor-suppressive activity (Ying et al., 2008).
- WNT5A promotes resistance of melanoma cell (Anastas et al., 2014).

Quantitative Understanding of the Linkage**Response-response relationship**

GSK3beta activity is inhibited by 1, 10, and 100 uM of LRP6 PPPSPxS peptides dose-dependently *in vitro* (Piao et al., 2008).

Time-scale

GSK3beta activity inhibition by LRP6 PPPSPxS peptides is measured in the reaction for 30 min at 37 °C *in vitro* (Piao et al., 2008).

Known modulating factors

FZD and LRP5/6 form dimers and Axin binds to the cytoplasmic tail of LRP5/6, which is phosphorylated by GSK3beta, followed by the inactivation of GSK3beta in Wnt/beta-catenin signaling (Mao et al., 2001) (He et al., 2004; Tamai et al., 2004; Zeng et al., 2005).

Axin is required for WNT3-induced FZD and LRP6 activation leading to the recruitment of GSK3beta to the plasma membrane (Zeng et al., 2008).

Known Feedforward/Feedback loops influencing this KER

The recruitment of GSK3beta together with Axin to LRP5/6 upon FZD activation decreases the phosphorylation of beta-catenin by GSK3beta (He et al., 2004; Tamai et al., 2004; Zeng et al., 2005).

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Relationship: 1926: GSK3 β inactivation leads to β -catenin activation (<https://aopwiki.org/relationships/1926>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Wnt ligand stimulation and Wnt signalling activation lead to cancer malignancy (https://aopwiki.org/aops/298)	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

GSK3-beta inhibition induced beta-catenin activation in human lung lymphatic endothelial cells (*Homo sapiens*) (Stump et al., 2019).

Key Event Relationship Description

GSK3beta inactivation leads to beta-catenin dephosphorylation, which avoids the ubiquitination of the beta-catenin and stabilize the beta-catenin (Clevers & Nusse, 2012).

Evidence Supporting this KER

Biological Plausibility

GSK3beta recruitment to LRP6 leads to form un-phosphorylated beta-catenin inducing the stabilization and translocation of the beta-catenin (MacDonald, Tamai, & He, 2009).

Stabilized beta-catenin accumulates in cytosol and translocates into the nucleus leading to beta-catenin activation (MacDonald et al., 2009).

Empirical Evidence

GSK3beta inactivation induces the beta-catenin stabilization (Pez et al., 2013).

GSK3beta inactivation induces beta-catenin translocation into the nucleus (MacDonald et al., 2009; Pez et al., 2013).

WNT2 knockdown induces the accumulation of GSK3beta in the cytoplasm and reduced the expression of beta-catenin, which WNT2 overexpression reduces the expression of GSK3beta in the cytoplasm and induces beta-catenin translocation into the nucleus (Wang, Li, & Kidder, 2010).

WNT2 siRNA knockdown increases the GSK3beta expression and decreases beta-catenin expression, and WNT2 overexpression reduces the GSK3beta and increases beta-catenin in granulosa cells in *Mus musculus* (Wang et al., 2010).

Uncertainties and Inconsistencies

GSK3beta phosphorylates LRP6 as well as remaining GSK3 beta phosphorylates beta-catenin which would be ubiquitinated and degraded (MacDonald et al., 2009).

Quantitative Understanding of the Linkage

Response-response relationship

GSK3beta inhibition by 1 mM of SB216763 or 5 mM of BRD3731 results in the decreased phosphorylation and stabilization of beta-catenin (Stump et al., 2019). The level of beta-catenin is increased by the inhibition of GSK3beta kinase activity (Stump et al., 2019). GSK3beta inhibition by small interference RNA (siRNA) of GSK3beta results in the decreased phosphorylation and increased expression of beta-catenin (Stump et al., 2019).

Time-scale

The treatment with SB216763 or BRD3731, GSK3beta inhibitors, decreases phosphorylated beta-catenin and increased beta-catenin expression in 48 hours (Stump et al., 2019). The cells are treated with GSK3beta small interference RNA (siRNA) for 48 hours to silence the expression of GSK3beta, which results in the activation of beta-catenin pathway (Stump et al., 2019).

Known modulating factors

Sortilin, a member of the sorting receptor family that transport intracellular proteins, regulates GSK3-beta, beta-catenin and Twist pathway activation to induce epithelial-mesenchymal transition and glioblastoma invasion (Yang et al., 2019).

Known Feedforward/Feedback loops influencing this KER

Beta-catenin is required and sufficient for the sequestration of GSK3 in acidic cytoplasmic endosomes (Taelman et al., 2010). Beta-catenin, of which level increases in Wnt signaling, facilitates GSK3 sequestration leading to feed-forward loop formation (Taelman et al., 2010).

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Relationship: 1927: β -catenin activation leads to Snail, Zeb, Twist activation (<https://aopwiki.org/relationships/1927>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Wnt ligand stimulation and Wnt signalling activation lead to cancer malignancy (https://aopwiki.org/aops/298)	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

The inhibition of c-MET decreases the expression of beta-catenin and Snail in human diffuse-type gastric cancer (Sohn et al., 2019).

The treatment with garcinol decreases the expression of beta-catenin and ZEB1/ZEB2 in human breast cancer cells (Ahmad et al., 2012).

Key Event Relationship Description

Beta-catenin activation, of which mechanism include the stabilization of the dephosphorylated beta-catenin and translocation of beta-catenin into the nucleus, induce the formation of beta-catenin-TCF complex and transcription of transcription factors such as Snail, Zeb and Twist (Clevers & Nusse, 2012) (Ahmad et al., 2012; Pearlman, Montes de Oca, Pal, & Afaq, 2017; Sohn et al., 2019; W. Yang et al., 2019).

Evidence Supporting this KER

Biological Plausibility

The treatment of human gastric cancer cells with INC280, which inhibits c-MET overexpressed in diffuse-type gastric cancer with poor prognosis, shows downregulation in beta-catenin and Snail expression, (Sohn et al., 2019).

The treatment with garcinol, a polyisoprenylated benzophenone derivative that is obtained from *Garcinia indica* extract, induced ZEB1 and ZEB2 down-regulation, increase in phosphorylated beta-catenin and decrease in nuclear beta-catenin in human breast cancer cells (Ahmad et al., 2012).

Sortilin, a member of the Vps10p sorting receptor family which is highly expressed in high-grade malignant glioma, positively regulates GSK-3beta/beta-catenin/Twist signaling pathway in glioblastoma (W. Yang et al., 2019).

Empirical Evidence

The inhibition of c-MET, which is overexpressed in diffuse-type gastric cancer, induced increase in phosphorylated beta-catenin, decrease in beta-catenin and Snail (Sohn et al., 2019).

The garcinol, that has anti-cancer effect, increases phosphorylated beta-catenin, decreases beta-catenin and ZEB1/ZEB2, and inhibit EMT (Ahmad et al., 2012).

The inhibition of sortilin by AF38469 (a sortilin inhibitor) or small interference RNA (siRNA) results in decrease in beta-catenin and Twist expression in human glioblastoma cells (W. Yang et al., 2019).

Uncertainties and Inconsistencies

It is possible that the inhibition of ZEB1 and ZEB2 by garcinol treatment is caused by down-regulation of NFkappaB and Wnt/beta catenin signaling (Ahmad et al., 2012).

Quantitative Understanding of the Linkage

Response-response relationship

The treatment with AF38469, a sortilin inhibitor, in 0, 100, 200, 400, 800, and 1600 nM concentration inhibited beta-catenin and Twist expression dose-dependently in human glioblastoma cells (W. Yang et al., 2019).

Time-scale

The treatment with 25 mM of garcinol for 48 hours induced increase in phosphorylated beta-catenin and decreased nuclear beta-catenin protein and ZEB1/ZEB2 mRNA in human breast cancer cells (Ahmad et al., 2012).

The treatment with AF38469, a sortilin inhibitor, for 0, 2, 4, 8, 16, or 24 hours shows that the expression of beta-catenin and Twist decrease in 8 hours followed by the subsequent decrease in 16 and 24 hours in human glioblastoma cells (W. Yang et al., 2019).

Known modulating factors

The proto-oncogene MET regulates beta-catenin and Snail expression (Sohn et al., 2019).

The inhibition of GSK3beta by SB216763 induced expression of beta-catenin and Twist, as well as mesenchymal markers such as N-cadherin, vimentin and MMP9 (W. Yang et al., 2019).

Known Feedforward/Feedback loops influencing this KER

The inhibited expression of phosphorylated GSK3beta, beta-catenin and Twist by sortilin inhibition is reversed by GSK3beta inhibition.

Furthermore, twist overexpression by lentivirus increased the inhibited expression of N-cadherin, MMP9 and vimentin and reverses the inhibitory effect of AF38469 on sortilin, which suggests that sortilin induces glioblastoma invasion mainly via GSK3beta/beta-catenin/Twist induced mesenchymal transition (W. Yang et al., 2019).

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Relationship: 1928: Snail, Zeb, Twist activation leads to Epithelial-mesenchymal transition (<https://aopwiki.org/relationships/1928>)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Wnt ligand stimulation and Wnt signalling activation lead to cancer malignancy (https://aopwiki.org/aops/298)	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Zeb1 activation leads to EMT via Prex1 activation in NCH421k, NCH441, and NCH644 human glioblastoma model cells (*Homo sapiens*) (Rosmaninho et al., 2018).

Zeb1 siRNA induced the suppression of EMT in SGC-7901 human gastric cancer cell line (*Homo sapiens*) (Xue et al., 2019).

Snail induces EMT in SAS and HSC-4 human head and neck squamous cancer cells (*Homo sapiens*) (Ota et al., 2016).

Snail induces EMT in B16-F10 murine melanoma cells (*Mus musculus*) (Kudo-Saito, Shirako, Takeuchi, & Kawakami, 2009; Y. Wang, Shi, Chai, Ying, & Zhou, 2013).

Twist1 is related to EMT in MCF-7 and MDA-MB-231 human breast cancer cell lines (*Homo sapiens*) (Menendez-Menendez et al., 2019). Hu et al., 2019).

Key Event Relationship Description

EMT-related transcription factors including Snail, ZEB and Twist are up-regulated in cancer cells (Diaz, Vinas-Castells, & Garcia de Herreros, 2014). The transcription factors such as Snail, ZEB and Twist bind to E-cadherin (CDH1) promoter and inhibit the CDH1 transcription via the consensus E-boxes (5'-CACCTG-3' or 5'-CAGGTG-3'), which leads to EMT (Diaz et al., 2014).

Evidence Supporting this KER**Biological Plausibility**

The transcription factors such as Snail, Zeb and Twist inhibit the CDH1 expression through their binding towards the promoter of CDH1, which leads to inhibition of cell adhesion and EMT (Diaz et al., 2014).

Empirical Evidence

Histone deacetylase inhibitors affect on EMT-related transcription factors including ZEB, Twist and Snail (Wawruszak et al., 2019).

Snail and Zeb induces EMT and suppress E-cadherin (CDH1) (Batlle et al., 2000; Diaz et al., 2014; Peinado, Olmeda, & Cano, 2007).

Uncertainties and Inconsistencies

The EMT is induced different transcription factors other than Zeb, Twist and Snail, which includes E47 and KLF8 (Diaz et al., 2014).

Zeb, Twist and Snail may activate or inactivate different genes or molecules to induce phenomena related to EMT and other phenomena other than EMT (Li & Balazsi, 2018).

Quantitative Understanding of the Linkage**Response-response relationship**

Snail (SNAI1) mRNA is methylated and N^6 -methyladenosine (m^6A) in its coding region (CDS) and 3' untranslated region (3'UTR) are significantly enriched during EMT progression (Lin et al., 2019). The m^6A enrichment fold of *SNAI1* mRNA in EMT cells is about 2.3-fold greater than in control cells (Lin et al., 2019).

Time-scale

Snail (SNAI1) transfection for 48 hours induce the repression of E-cadherin (CDH1) protein expression (Lin et al., 2019).

SNAI1 mRNA in polysome is up-regulated in EMT-undergoing HeLa cells treated with 10 ng/ml of TGF-beta for 3 days compared with control cells (Lin et al., 2019).

Known modulating factors

The decrease in E-cadherin (CDH1), a cell adhesion molecule, is related to EMT (Diaz et al., 2014).

Methyltransferase-like 3 (METTL3) modulates methylation of Snail (SNAI1) mRNA and EMT (Lin et al., 2019).

Binding of beta-catenin to members of the TCF/LEF family transcription factors increase gene expression related to EMT such as Twist and decrease E-cadherin protein expression (Qualtrough, Rees, Speight, Williams, & Paraskeva, 2015).

Known Feedforward/Feedback loops influencing this KER

The inhibition of Hedgehog signaling pathway with cyclopamine reduces beta-catenin-TCF transcriptional activity, decreases the Twist expression, induces E-cadherin expression and inhibits EMT (Qualtrough et al., 2015).

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Relationship: 1929: Epithelial-mesenchymal transition leads to Cancer Malignancy
(https://aopwiki.org/relationships/1929)

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Wnt ligand stimulation and Wnt signalling activation lead to cancer malignancy (https://aopwiki.org/aops/298)	adjacent	High	Low

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=9606)

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

EMT induces cancer invasion, metastasis (*Homo sapiens*)(P. Zhang et al., 2015).

EMT is related to cancer drug resistance in MCF-7 human breast cancer cells (*Homo sapiens*)(B. Du & Shim, 2016).

Key Event Relationship Description

Some population of the cells exhibiting EMT demonstrates the feature of cancer stem cells (CSCs), which are related to cancer malignancy (Shibue & Weinberg, 2017; Shihori Tanabe, 2015a, 2015b; Tanabe, Aoyagi, Yokozaki, & Sasaki, 2015).

EMT phenomenon is related to cancer metastasis and cancer therapy resistance (Smith & Bhowmick, 2016; Tanabe, 2013). Increase expression of enzymes that degrade the extracellular matrix components and the decrease in adhesion to the basement membrane in EMT induce the cell escape from the basement membrane and metastasis (Smith & Bhowmick, 2016). Morphological changes observed during EMT is associated with therapy resistance (Smith & Bhowmick, 2016).

Evidence Supporting this KER

Biological Plausibility

The morphological and physiological changes associated with EMT are involved in invasiveness and drug resistance (Shibue & Weinberg, 2017). The EMT-activated particular carcinoma cells in primary tumors invade the surrounding stroma (Shibue & Weinberg, 2017). The EMT – activated carcinoma cells interact with the surrounding extracellular matrix protein to induce focal adhesion kinase and extracellular signal-related kinase activation, followed by the transforming growth factor beta (TGFbeta) and canonical and/or noncanonical Wnt pathways to induce cancer stem cell (CSC) properties which contribute to the drug resistance (Shibue & Weinberg, 2017).

EMT-associated down-regulation of multiple apoptotic signaling pathways induce drug efflux and slow cell proliferation to induce the general resistance of carcinoma cells to anti-cancer drugs (Shibue & Weinberg, 2017).

Snail, an EMT-related transcription factor, induces the expression of the AXL receptor tyrosine kinase, which enables the cancer cells to survive by the activation of AXL signaling triggered by the binding of its ligand growth arrest-specific protein 6 (GAS6)(Shibue & Weinberg, 2017).

The EMT-activated cells evade the lethal effect of cytotoxic T cells, which include the elevated expression of programmed cell death 1 ligand (PD-L1) which binds to the programmed cell death protein 1 (PD-1) inhibitory immune-checkpoint receptor on the cell surface of cytotoxic T cells (Shibue & Weinberg, 2017).

Empirical Evidence

Slug/Snai2, a *ces-1*-related zinc finger transcription factor gene, confers resistance to p53-mediated apoptosis of hematopoietic progenitors by repressing *PUMA* (also known as *BBC3*, encoding Bcl-2-binding component 3) (Inukai et al., 1999; Shibue & Weinberg, 2017; W.-S. Wu et al., 2005).

EMT activation induces the expression of multiple members of the ATP-binding cassette (ABC) transporter family, which results in the resistant to doxorubicin (Saxena, Stephens, Pathak, & Rangarajan, 2011; Shibue & Weinberg, 2017)

TGFbeta-1 induced EMT results in the acquisition of cancer stem cell (CSC) like properties (Pirozzi et al., 2011; Shibue & Weinberg, 2017).

Snail-induced EMT induces the cancer metastasis and resistance to dendritic cell-mediated immunotherapy (Kudo-Saito, Shirako, Takeuchi, & Kawakami, 2009).

Zinc finger E-box-binding homeobox (ZEB1)-induced EMT results in the relief of miR-200-mediated repression of programmed cell death 1 ligand (PD-L1) expression, a major inhibitory ligand for the programmed cell death protein (PD-1) immune-checkpoint protein on CD8+ cytotoxic T lymphocyte (CTL), subsequently the CD8+ T cell immunosuppression and metastasis (Chen et al., 2014).

Uncertainties and Inconsistencies

The reversing process of EMT, which names as mesenchymal-epithelial transition (MET), may be one of the candidates for the anti-cancer therapy, where the plasticity of the cell phenotype is of importance and under investigation (Shibue & Weinberg, 2017).

Quantitative Understanding of the Linkage

Response-response relationship

Induction of EMT by TGFbeta and Twist increase the gene expression of EMT markers such as Snail, Vimentin, N-cadherin, and ABC transporters including ABCA3, ABCC1, ABCC3 and ABCC10 (Saxena et al., 2011).

Human mammary epithelial cells (HMLE) stably expressing Twist, FOXC2 or Snail demonstrates the increased the cell viability compared to control HMLE in the treatment with about 0.3, 3, 30 mM of doxorubicin, dose-dependently (Saxena et al., 2011).

Time-scale

The treatment with doxorubicin for 48 hours demonstrates the increase in the cell viability in Twist/FOXC2/Snail overexpressed HMLE compared to control HMLE (Saxena et al., 2011).

The inhibition of Twist or Zeb1 with small interference RNA (siRNA) induced the inhibition of the cell viability compared to control MDAMB231 cells treated with doxorubicin for 48 hours (Saxena et al., 2011).

Known modulating factors

ABC transporters which are related to drug resistance are overexpressed in the EMT-activated cells (Saxena et al., 2011). The expression of PD-L1, which binds to the PD-1 on the cytotoxic T cells, is up-regulated in EMT-activated cells, which results in the inhibition of cancer immunity and the resistance to cancer therapy (Shibue & Weinberg, 2017).

Known Feedforward/Feedback loops influencing this KER

The investigation of EMT-CSC relations is important to understand the relationship between EMT and cancer malignancy. Non-CSCs in cancer can spontaneously undergo EMT and dedifferentiate into new CSC, subsequently induce the regeneration of tumorigenic potential (Marjanovic, Weinberg, & Chaffer, 2013; Shibue & Weinberg, 2017).

The plastic CSC theory demonstrates the bidirectional conversions between non-CSCs and CSCs, which may contribute into the acquisition of cancer malignancy in EMT-activated cells (Marjanovic et al., 2013).

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